OPENING PANDORA'S BOX: CHARACTERIZATION OF THE DISTRIBUTION, AND ACTIVITY OF THE SALMONELLA CYTOLETHAL DISTENDING TOXIN AMONG NONTYPHOIDAL *SALMONELLA* SEROTYPES

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Rachel Alessandra Miller

August 2017

OPENING PANDORA'S BOX: CHARACTERIZATION OF THE DISTRIBUTION AND ACTIVITY OF THE SALMONELLA CYTOLETHAL DISTENDING TOXIN AMONG NONTYPHOIDAL *SALMONELLA* SEROTYPES

Rachel Alessandra Miller, Ph.D.

Cornell University 2017

Salmonella enterica subsp. *enterica* is one of the leading causes of foodborne illness worldwide. Recently, genomic characterizations of *S.* Typhi, the causative agent of typhoid fever, identified a novel hybrid toxin which combines the active subunits of both the pertussis toxin and the cytolethal distending toxin, into one holotoxin which was called the 'typhoid toxin', for its hypothesized role in typhoid fever. However, the 'typhoid toxin' was later found to be encoded by several nontyphoidal serotypes as well, and therefore will be referred to as S-CDT (for Salmonella Cytolethal Distending Toxin). The overall goal of the work presented here was to characterize the 'typhoid toxin' encoded by nontyphoidal serotypes, including i) the distribution and conservation of S-CDT genes in common nontyphoidal serotypes, (ii) characterization of the activity and function of the toxin at the cellular level, and (iii) asserting which genes are essential for toxin activity *in vitro*.

Among the nontyphoidal serotypes causing roughly 75% of human cases of salmonellosis in the US, genes encoding S-CDT were conserved in (50 out of 50 isolates screened) serotypes Javiana, Montevideo, and Oranienburg, while for serotype Mississippi, S-CDT genes were clade-associated. Alignment of amino acid sequences of S-CDT subunits demonstrated that these 4 nontyphoidal serotypes encoded an S-CDT with high similarity to the typhoid toxin encoded by serotype Typhi. Infection of eukaryotic cells with S-CDT-positive serotypes, but not S-CDT-

negative serotypes resulted in activation of the DNA damage response, and a G2/M cell cycle arrest, both of which support that these serotypes produce an active toxin. This activity was dependent on CdtB, the active subunit of S-CDT which acts as a nuclease *in vitro*, as deletion of *cdtB* abolished the ability of the S-CDT positive serotypes to activate the DNA damage response.

Similar to Typhi, deletion of toxin subunit genes *cdtB* and *pltA* abolished activity *in vitro*. In contrast, deletion of *pltB*, encoding the binding subunit, did not affect S-CDT intoxication, suggesting that either the trafficking or activity of S-CDT differs between nontyphoidal and typhoidal serotypes, or that the toxin uses an alternative binding subunit. Genetic screening showed that *S.* Javiana (a nontyphoidal serotype) encoded a gene, *artB*, with homology to *pltB*. Deletion of *artB* decreased, but did not eliminate, S-CDT activity *in vitro*, but deletion of both *artB* and *pltB* abolished S-CDT-induced activity, suggesting that at least one subunit is required for S-CDT activity *in vitro*. Strains harboring deletions in either *ttsA* or *STY1887* did not have a significant effect on S-CDT-mediated intoxication, suggesting that they are not essential for activity *in vitro*.

Importantly, this work provides experimental evidence that nontyphoidal serotypes not only encode a 'typhoid toxin' that is nearly identical at the amino acid level to the one encoded by *S.* Typhi, but that it is also functional in these serotypes. Furthermore, we provide evidence that S-CDT positive nontyphoidal serotypes significantly impact the outcome of infection at the cellular level. Taken together, these results suggest that S-CDT plays an important role in nontyphoidal salmonellosis, and provides one such 'factor' that may aide in current efforts to target specific serotypes for reducing the incidence of human cases of salmonellosis in the US.

BIOGRAPHICAL SKETCH

"Life is a journey, not a destination." Rachel began her journey in Fort Ord, California. As the daughter of a veterinarian (mother) and a meteorologist (father), scientific discovery was instilled into her at a very young age. Rachel earned Bachelor of Science degrees in Food Science and Microbiology from Oregon State University, where she completed an Honors thesis examining the impact of light backscattering on the physical properties of milk coagulation under the mentorship of Dr. Lisbeth Goddik. From there, Rachel spent 2 months researching novel processing techniques for ice cream and learning the art of artisan cheese making in Poligny, France. Rachel began her graduate studies in Dr. Martin Wiedmann's lab in 2012. Throughout her graduate experience, international research became a recurring theme for Rachel. Rachel had the opportunity to spend a total of 8 months in Nairobi, Kenya as a Borlaug Fellow, working on characterizing the importance of surface glycosylation in the cattle and goat pathogen *Mycoplasma mycoides*. Rachel also traveled to Ethiopia, France, and China to attend conferences and short research-related training experiences. After completing her dissertation, Rachel will continue her journey as a postdoctoral researcher at the University of British Columbia in Vancouver, BC. Rachel aspires to become a faculty member, which she hopes will enable her to pursue her passions of teaching and research.

For my Mom and Dad, whose unwavering encouragement and support helped me to realize that dreams can come true

ACKNOWLEDGEMENTS

I once heard that it ‘takes a village to raise a child.’ I thoroughly believe that it takes several ‘villages’ to complete a dissertation. With that being said, I have a number of outstanding individuals to recognize, who, without their assistance, this dissertation never would have come to fruition.

First, I would like to thank my advisor Dr. Martin Wiedmann. I cannot express how grateful I am to have had the opportunity to work with Martin for the last 5 years. Martin gave me the flexibility and freedom to dream up my own research ideas, and translate them from “I wonder what would happen if…” experiments into meaningful manuscripts. I still don’t understand how someone who is that busy, could still find time to squeeze in a 30-minute impromptu meeting to help me decide between 2 experimental designs, or whatever else I was struggling with on a given day.

Dr. Craig Altier was a tremendous resource and collaborator for all of the *Salmonella* work. I thoroughly enjoyed having the opportunity to run through experimental ideas with him, and just ‘talk science.’ I am also extremely grateful for the experimental advice and assistance provided by his lab members Dr. Colleen Eade, Dr. Chien-che Hung, and Dr. Michael Betteken. I would also like to extend a sincere thank you to Dr. Motoko Mukai who served as my minor advisor for my environmental toxicology minor.

Throughout my graduate experience, I had the opportunity to work with a number of faculty members who all ‘unofficially’ mentored me. I would like to especially thank Dr. Dennis Miller and Dr. Cy Lee, who taught me a lot about life in academia and beyond.

Nobody ever said that a Ph.D. was an easy endeavor. But knowing that you were in good company made it so much more enjoyable. For this reason, I would like to thank my cohort

members for their support: Dr. Yifan Cheng, Dr. Abby Snyder (and her dog, Little Abby, who always had the best birthday parties), Dr. Claire Zoellner, Dr. Charles Lee, Dr. Ezen Choo, and Dr. Andrew Kauffmann.

I would also like to thank my Food Safety Lab (FSL) family. Dr. Claudia Guldimann was a tremendous help teaching me the ropes of tissue culture, flow cytometry, immunofluorescence staining, and all around, just a really great role model. Maureen Gunderson prepared thousands of plates, bottles of media, and kept everything organized in the lab. Sherry Roof and Nicole Martin were both phenomenal lab managers, and kept us all in line. And former and present members of the FSL, especially: Steve Warchocki, Aljosa Trmcic, Jeff Tokman, Sandy Alles, Dan Weller, David Kent, Ariel Buehler, Sam Reichler, Sean Guo, Shelley Clark, Dr. Veronica Guariglia-Oropeza, Dr. Ahmed Gaballa, Laura Carroll, Rachel Evanowski, and Sarah Beno.

The students that I had the opportunity to mentor, and who, in turn, mentored me about being a mentor: Miqueala Hanselmann, Lang Sun, Jiahui Jian, Sarah Kozak, and Clint Hervert.

And last, but certainly not least, I would like to thank my friends and family. My parents, Ron and Sylvia, who have always given me encouragement, love, and support. And a special thank you to; Dr. Jasna Kovac, who was my travel partner, gym buddy, and a great scientist and an amazing friend; Lory Henderson (LOH), who always brightens up the room with her smiles, hugs, laughs, and mad dance moves; Mee-ya Monnin, my pseudo-sister and best friend; and Graceful Chang-Chang (Yichang Lui), Smart Qiu-Qiu (Jingqiu Liao), and Beautiful Lang-Lang (Lang Sun).

Merci beaucoup et meilleurs vœux~
Asante sana na kila la heri~
Thank you and best wishes.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

Foodborne illness sickens an estimated 1 in 6 people in the US each year, accounting for an estimated 48 million illnesses (90% credible interval: 28.7 - 71.1 million) and 3,037 deaths (90% credible interval: 1,492 – 4,983) per year (1). Economically, this equates to over $14.0 billion (90% credible interval: $4.4 - $33.0 billion USD) accrued from the cost of illness (2). The foodborne pathogen *Salmonella enterica* subspecies *enterica* is the leading cause of foodborne illness resulting from a bacterial agent, in the US (3), and is the second leading agent worldwide (4). As such, an improved understanding of the virulence mechanisms employed by *S. enterica* represents a key component in understanding the nature of salmonellosis, in order to reduce morbidity and mortality associated with this foodborne pathogen. The overall goal of this dissertation was to assess the importance of the cytolethal distending toxin (CDT), a toxin which has been previously associated with DNA damage resulting from infection with other bacterial pathogens (5-7), in the context of foodborne salmonellosis.

Significance of foodborne nontyphoidal salmonellosis. In the US foodborne infections with nontyphoidal *Salmonella* (NTS; i.e., all serotypes except for Typhi) account for an estimated 1.03 million illnesses, 19,336 hospitalizations, and 378 deaths annually (3), the most of any foodborne pathogen in the US. Globally, the impact of nontyphoidal salmonellosis is much greater, causing an estimated 93.8 million cases, and 155,000 deaths (8).

***Salmonella* is a Gram-negative foodborne pathogen.** The genus *Salmonella* includes 2 species, *enterica* and *bongori* (9). Within the species *enterica*, there are 6 subspecies; *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica*. The majority of *Salmonella* infecting warm-blooded hosts belong to subspecies *enterica*. Characterization of the O (somatic) and H (flagellar)

antigens using anti-sera (called serotyping) was adopted as a classification scheme among *Salmonella* subspecies. Currently, there are over 2,600 serotypes (10), but in the U.S., the top 20 most commonly isolated serotypes among subspecies *enterica* account for >85% of all NTS infections (CDC, 2011).

Infection with typhoidal and paratyphoidal serotypes (i.e. *S. enterica* subsp. *enterica* serotypes Typhi, Paratyphi A, Paratyphi B, and Paratyphi C) typically results in a more severe, prolonged illness characterized by a high fever (>39°C), malaise, vomiting, headache, and an elevated pulse rate (11). Serotype Typhi is a host-restricted serotype, and is transmitted from human to human, primarily through contamination of drinking water or improper food handling (12). Infections with nontyphoidal serotypes (NTS; i.e. all serotypes not Typhi, Paratyphi A, Paratyphi B, or Paratyphi C) typically result in a self-limiting gastroenteritis that is short-lived (13). However, NTS vary greatly with respect to the severity of the illness, with certain serotypes being significantly more likely to result in invasive disease, hospitalization, and death (14). Genomic and phenotypic profiling of NTS have expanded our understanding of *Salmonella* virulence, but these efforts have failed to definitively account for differences in disease severity (13, 15, 16). Nontyphoidal salmonellosis has been linked to several long term sequelae including reactive arthritis and post-infectious irritable bowel syndrome (17), although the exact mechanisms behind the development of these long-term sequelae remain unknown. Therefore, characterizations of novel virulence factors produced by NTS are of critical importance in understanding why certain nontyphoidal salmonellosis cases differ in severity, and are more likely to result in long term sequelae.

The cytolethal distending toxin is an A-B type toxin associated with eukaryotic cell DNA damage. The cytolethal distending toxin (CDT) was first reported by Johnson and Lior, who

observed that the addition of supernatants of *Escherichia coli* to CHO cell monolayers resulted in a distended cell morphology, G2/M cell cycle arrest, and eventually, cell death (18). The number of species encoding a CDT has expanded to 8 bacterial genera including, *Campylobacter* spp. (19-22) *Haemophilus* spp. (23-25), *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) (26, 27), *Helicobacter* spp. (28-30), *Shigella* spp. (31), *Yersinia* spp. (32), *Providencia alcalifaciens* (33), and *Salmonella* (34, 35). Interestingly, the CDT produced by all genera, except for *Salmonella*, exists as a tripartite toxin consisting of equimolar ratios of binding and trafficking components CdtA and CdtC, and the active component CdtB (5, 6, 36). CdtB was originally characterized as a nuclease, owing to its structural homology to Dnase I, and its activity as a nuclease in a super-coiled plasmid assay (36). The CDT produced by *A. actinomycetemcomitans* also has phosphatidylinositol-3,4,5-triphosphate phosphatase activity (37), which could interfere with host signaling pathways (38).

Importance of characterizing S-CDT as a novel virulence factor in nontyphoidal salmonellosis. The CDT encoded by *Salmonella* (called S-CDT) was first characterized in *S.* Typhi, and was thought to be unique to this serotype (34, 35, 39). S-CDT is distinct from CDTs produced by other Gram-negative pathogens, as it substitutes the binding subunits CdtA and CdtC, for the active and binding subunits of the pertussis toxin (5, 32, 34, 40). Genomic characterizations of NTS have since established that this novel virulence factor is present in at least 40 NTS as well (15, 40). Importantly, administration of purified S-CDT was shown to recapitulate symptoms of typhoid fever, lending to its classification as the "typhoid toxin" (41). The DNA damaging activity of S-CDT, the hallmark of the CDT family of toxins (5, 32), has been established both *in vitro* and *in vivo* for *S.* Typhi (41, 42). Furthermore, recent research established a role for S-CDT in prolonged infections with *S.* Typhi, demonstrating the

importance of the toxin in immune evasion (42). It was recently established that S-CDT plays an important role in infections with NTS serotypes as well, significantly altering the outcome of infection at the cellular level (43).

Significance of bacterial genotoxins in development of cancer. CDTs have been associated with an increased risk of genomic instability (44). Causal links between bacterial genotoxins and cancer have been established for *E. coli* (45, 46), and *Helicobacter pylori* (47). Furthermore, chronic infection with *S.* Typhi is associated with an increased risk of gallbladder cancer (48-51). Although the potential role of S-CDT in the development of gallbladder cancer, has not been established, chronic exposure to CDT has been shown to cause genomic instability leading to anchorage-independent growth [i.e. malignant transformation (44)]. Little is known about the activity of S-CDT *in vitro,* with regards to the type of DNA damage induced, how the DNA damage is repaired, or the ultimate outcome of S-CDT-mediated DNA damage. Detailed characterizations of the DNA damage invoked by exposure to S-CDT produced by NTS, along with the ultimate fate of S-CDT intoxicated cells is critical to assessing the potential contributions of S-CDT to carcinogenesis.

Overall, our knowledge of the contributions of S-CDT to acute and chronic sequelae resulting from nontyphoidal salmonellosis, is very limited. The ultimate goal of this dissertation was to understand the distribution, regulation, and contributions of S-CDT to salmonellosis. The following chapters address key knowledge gaps in our understanding of S-CDT. Namely, this dissertation (i) provides a review of the literature characterizing S-CDT from both typhoidal and nontyphoidal serotypes, (ii) describes the distribution and activity of S-CDT encoded by nontyphoidal serotypes associated with foodborne salmonellosis in the US, and (iii) expands our

understanding of the genetic requirements for S-CDT-mediated intoxication of normal human intestinal epithelial cells.

REFERENCES

1. Scallan E, Griffin PM, Angulo FJ, Tauxe RV, Hoekstra RM. 2011. Foodborne illness acquired in the United States--unspecified agents. Emerg Infect Dis 17:16-22.
2. Hoffmann S, Maculloch B, Batz M. 2015. Economic burden of major foodborne illnesses acquired in the United States. Current Politics and Economics of the United States, Canada and Mexico 17:543.
3. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson M-A, Roy SL, Jones JL, Griffin PM. 2011. Foodborne illness acquired in the United States—major pathogens. Emerg Infect Dis 17.
4. Kirk MD, Pires SM, Black RE, Caipo M, Crump JA, Devleesschauwer B, Döpfer D, Fazil A, Fischer-Walker CL, Hald T, Hall AJ, Keddy KH, Lake RJ, Lanata CF, Torgerson PR, Havelaar AH, Angulo FJ. 2015. World Health Organization Estimates of the Global and Regional Disease Burden of 22 Foodborne Bacterial, Protozoal, and Viral Diseases, 2010: A Data Synthesis. PLoS Med 12:e1001921.
5. Jinadasa RN, Bloom SE, Weiss RS, Duhamel GE. 2011. Cytolethal distending toxin: a conserved bacterial genotoxin that blocks cell cycle progression, leading to apoptosis of a broad range of mammalian cell lineages. Microbiology 157:1851-1875.
6. Guerra L, Cortes-Bratti X, Guidi R, Frisan T. 2011. The biology of the cytolethal distending toxins. Toxins 3:172-190.
7. Bezine E, Vignard J, Mirey G. 2014. The cytolethal distending toxin effects on mammalian cells: a DNA damage perspective. Cells 3:592-615.
8. Majowicz SE, Musto J, Scallan E, Angulo FJ, Kirk M, O'Brien SJ, Jones TF, Fazil A, Hoekstra RM. 2010. The global burden of nontyphoidal *Salmonella* gastroenteritis. Clinical Infectious Diseases 50:882-889.
9. Brenner F, Villar R, Angulo F, Tauxe R, Swaminathan B. 2000. Salmonella nomenclature. Journal of clinical microbiology 38:2465-2467.
10. Issenhuth-Jeanjean S, Roggentin P, Mikoleit M, Guibourdenche M, de Pinna E, Nair S, Fields PI, Weill F-X. 2014. Supplement 2008–2010 (no. 48) to the White–Kauffmann–Le Minor scheme. Research in Microbiology 165:526-530.
11. Dougan G, Baker S. 2014. Salmonella enterica serovar Typhi and the pathogenesis of typhoid fever. Annual review of microbiology 68:317-336.
12. Connor BA, Schwartz E. 2005. Typhoid and paratyphoid fever in travellers. The Lancet infectious diseases 5:623-628.
13. Gal-Mor O, Boyle EC, Grassl GA. 2014. Same species, different diseases: how and why typhoidal and non-typhoidal Salmonella enterica serovars differ. Frontiers in microbiology 5:391.
14. Jones TF, Ingram LA, Cieslak PR, Vugia DJ, Tobin-D'Angelo M, Hurd S, Medus C, Cronquist A, Angulo FJ. 2008. Salmonellosis outcomes differ substantially by serotype. Journal of Infectious Diseases 198:109-114.
15. den Bakker HC, Switt AIM, Govoni G, Cummings CA, Ranieri ML, Degoricija L, Hoelzer K, Rodriguez-Rivera LD, Brown S, Bolchacova E. 2011. Genome sequencing reveals diversification of virulence factor content and possible host adaptation in distinct subpopulations of *Salmonella enterica*. BMC genomics 12:425.
16. Suez J, Porwollik S, Dagan A, Marzel A, Schorr YI, Desai PT, Agmon V, McClelland M, Rahav G, Gal-Mor O. 2013. Virulence gene profiling and pathogenicity characterization

of non-typhoidal *Salmonella* accounted for invasive disease in humans. PLoS One 8:e58449.
17. Connor BA, Riddle MS. 2013. Post-infectious sequelae of travelers' diarrhea. Journal of travel medicine 20:303-312.
18. Johnson W, Lior H. 1987. Response of Chinese hamster ovary cells to a cytolethal distending toxin (CDT) of *Escherichia coli* and possible misinterpretation as heat-labile (LT) enterotoxin. FEMS microbiology letters 43:19-23.
19. Whitehouse CA, Balbo PB, Pesci EC, Cottle DL, Mirabito PM, Pickett CL. 1998. *Campylobacter jejuni* cytolethal distending toxin causes a G2-phase cell cycle block. Infection and immunity 66:1934-1940.
20. Mooney A, Clyne M, Curran T, Doherty D, Kilmartin B, Bourke B. 2001. *Campylobacter upsaliensis* exerts a cytolethal distending toxin effect on HeLa cells and T lymphocytes. Microbiology 147:735-743.
21. Hickey TE, Majam G, Guerry P. 2005. Intracellular survival of *Campylobacter jejuni* in human monocytic cells and induction of apoptotic death by cytholethal distending toxin. Infection and immunity 73:5194.
22. Asakura M, Samosornsuk W, Taguchi M, Kobayashi K, Misawa N, Kusumoto M, Nishimura K, Matsuhisa A, Yamasaki S. 2007. Comparative analysis of cytolethal distending toxin (cdt) genes among *Campylobacter jejuni, C. coli* and *C. fetus* strains. Microbial pathogenesis 42:174-183.
23. Wising C, Azem J, Zetterberg M, Svensson LA, Ahlman K, Lagergård T. 2005. Induction of apoptosis/necrosis in various human cell lineages by *Haemophilus ducreyi* cytolethal distending toxin. Toxicon 45:767-776.
24. Gargi A, Tamilselvam B, Powers B, Prouty MG, Lincecum T, Eshraghi A, Maldonado-Arocho FJ, Wilson BA, Bradley KA, Blanke SR. 2013. Cellular interactions of the cytolethal distending toxins from *Escherichia coli* and *Haemophilus ducreyi*. Journal of Biological Chemistry 288:7492-7505.
25. Dixon SD, Huynh MM, Tamilselvam B, Spiegelman LM, Son SB, Eshraghi A, Blanke SR, Bradley KA. 2015. Distinct Roles for CdtA and CdtC during Intoxication by Cytolethal Distending Toxins. PLoS ONE 10:e0143977.
26. Rabin SD, Flitton JG, Demuth DR. 2009. *Aggregatibacter actinomycetemcomitans* cytolethal distending toxin induces apoptosis in nonproliferating macrophages by a phosphatase-independent mechanism. Infection and immunity 77:3161-3169.
27. Damek-Poprawa M, Jang JY, Volgina A, Korostoff J, DiRienzo JM. 2012. Localization of *Aggregatibacter actinomycetemcomitans* cytolethal distending toxin subunits during intoxication of live cells. Infection and immunity 80:2761-2770.
28. Young VB, Knox KA, Schauer DB. 2000. Cytolethal distending toxin sequence and activity in the enterohepatic pathogen *Helicobacter hepaticus*. Infection and immunity 68:184-191.
29. Pratt JS, Sachen KL, Wood HD, Eaton KA, Young VB. 2006. Modulation of host immune responses by the cytolethal distending toxin of *Helicobacter hepaticus*. Infection and immunity 74:4496-4504.
30. Liyanage NP, Manthey KC, Dassanayake RP, Kuszynski CA, Oakley GG, Duhamel GE. 2010. *Helicobacter hepaticus* cytolethal distending toxin causes cell death in intestinal epithelial cells via mitochondrial apoptotic pathway. Helicobacter 15:98-107.

31. Okuda J, Fukumoto M, Takeda Y, Nishibuchi M. 1997. Examination of diarrheagenicity of cytolethal distending toxin: suckling mouse response to the products of the *cdtABC* genes of *Shigella dysenteriae*. Infection and immunity 65:428-433.
32. Gargi A, Reno M, Blanke SR. 2012. Bacterial toxin modulation of the eukaryotic cell cycle: are all cytolethal distending toxins created equally? Frontiers in cellular and infection microbiology 2.
33. Asakura H, Momose Y, Ryu C-H, Kasuga F, Yamamoto S, Kumagai S, Igimi S. 2013. *Providencia alcalifaciens* causes barrier dysfunction and apoptosis in tissue cell culture: potent role of lipopolysaccharides on diarrheagenicity. Food Additives & Contaminants: Part A 30:1459-1466.
34. Spanò S, Ugalde JE, Galán JE. 2008. Delivery of a *Salmonella* Typhi exotoxin from a host intracellular compartment. Cell host & microbe 3:30-38.
35. Haghjoo E, Galán JE. 2004. *Salmonella* Typhi encodes a functional cytolethal distending toxin that is delivered into host cells by a bacterial-internalization pathway. Proceedings of the National Academy of Sciences of the United States of America 101:4614-4619.
36. Hu X, Nesic D, Stebbins CE. 2006. Comparative structure-function analysis of cytolethal distending toxins. Proteins 62:421-434.
37. Shenker BJ, Dlakić M, Walker LP, Besack D, Jaffe E, LaBelle E, Boesze-Battaglia K. 2007. A novel mode of action for a microbial-derived immunotoxin: the cytolethal distending toxin subunit B exhibits phosphatidylinositol 3, 4, 5-triphosphate phosphatase activity. The Journal of Immunology 178:5099-5108.
38. Scuron MD, Boesze-Battaglia K, Dlakić M, Shenker BJ. 2016. The Cytolethal Distending Toxin Contributes to Microbial Virulence and Disease Pathogenesis by Acting As a Tri-Perditious Toxin. Frontiers in Cellular and Infection Microbiology 6:168.
39. Parkhill J, Dougan G, James K, Thomson N, Pickard D, Wain J, Churcher C, Mungall K, Bentley S, Holden M. 2001. Complete genome sequence of a multiple drug resistant *Salmonella enterica* serovar Typhi CT18. Nature 413:848-852.
40. Miller R, Wiedmann M. 2016. Dynamic Duo—The Salmonella Cytolethal Distending Toxin Combines ADP-Ribosyltransferase and Nuclease Activities in a Novel Form of the Cytolethal Distending Toxin. Toxins 8:121.
41. Song J, Gao X, Galán JE. 2013. Structure and function of the *Salmonella* Typhi chimaeric A2B5 typhoid toxin. Nature 499:350-354.
42. Belluz LDB, Guidi R, Pateras IS, Levi L, Mihaljevic B, Rouf SF, Wrande M, Candela M, Turroni S, Nastasi C. 2016. The Typhoid Toxin Promotes Host Survival and the Establishment of a Persistent Asymptomatic Infection. PLoS Pathog 12:e1005528.
43. Rodriguez-Rivera LD, Bowen BM, den Bakker HC, Duhamel GE, Wiedmann M. 2015. Characterization of the cytolethal distending toxin (typhoid toxin) in non-typhoidal *Salmonella* serovars. Gut pathogens 7:1.
44. Guidi R, Guerra L, Levi L, Stenerlöw B, Fox JG, Josenhans C, Masucci MG, Frisan T. 2013. Chronic exposure to the cytolethal distending toxins of Gram-negative bacteria promotes genomic instability and altered DNA damage response. Cellular microbiology 15:98-113.
45. Buc E, Dubois D, Sauvanet P, Raisch J, Delmas J, Darfeuille-Michaud A, Pezet D, Bonnet R. 2013. High prevalence of mucosa-associated *E. coli* producing cyclomodulin and genotoxin in colon cancer. PLoS One 8:e56964.

46. Cougnoux A, Dalmasso G, Martinez R, Buc E, Delmas J, Gibold L, Sauvanet P, Darcha C, Déchelotte P, Bonnet M. 2014. Bacterial genotoxin colibactin promotes colon tumour growth by inducing a senescence-associated secretory phenotype. Gut:gutjnl-2013-305257.
47. Toller IM, Neelsen KJ, Steger M, Hartung ML, Hottiger MO, Stucki M, Kalali B, Gerhard M, Sartori AA, Lopes M. 2011. Carcinogenic bacterial pathogen *Helicobacter pylori triggers* DNA double-strand breaks and a DNA damage response in its host cells. Proceedings of the National Academy of Sciences 108:14944-14949.
48. Gonzalez-Escobedo G, Marshall JM, Gunn JS. 2011. Chronic and acute infection of the gall bladder by *Salmonella* Typhi: understanding the carrier state. Nature Reviews Microbiology 9:9-14.
49. Gunn JS, Marshall JM, Baker S, Dongol S, Charles RC, Ryan ET. 2014. *Salmonella* chronic carriage: epidemiology, diagnosis, and gallbladder persistence. Trends in microbiology 22:648-655.
50. Nagaraja V, Eslick G. 2014. Systematic review with meta-analysis: the relationship between chronic *Salmonella* Typhi carrier status and gall-bladder cancer. Alimentary pharmacology & therapeutics 39:745-750.
51. Scanu T, Spaapen RM, Bakker JM, Pratap CB, Wu L-e, Hofland I, Broeks A, Shukla VK, Kumar M, Janssen H. 2015. *Salmonella* Manipulation of Host Signaling Pathways Provokes Cellular Transformation Associated with Gallbladder Carcinoma. Cell Host & Microbe.

CHAPTER 2

DYNAMIC DUO- THE SALMONELLA CYTOLETHAL DISTENDING TOXIN COMBINES ADP-RIBOSYLTRANSFERASE AND NUCLEASE ACTIVITIES IN A NOVEL FORM OF THE CYTOLETHAL DISTENDING TOXIN*

*****R.A. Miller, and M. Wiedmann

Published in Toxins 2016, 8, 121; doi:10.3390/toxins8050121

ABSTRACT

The cytolethal distending toxin (CDT) is a well characterized bacterial genotoxin encoded by several Gram-negative bacteria, including *Salmonella enterica* (*S. enterica*). The CDT produced by *Salmonella* (S-CDT) differs from the CDT produced by other bacteria, as it utilizes subunits with homology to the pertussis and subtilase toxins, in place of the traditional CdtA and CdtC subunits. Previously, S-CDT was thought to be a unique virulence factor of *S. enterica* subspecies *enterica* serotype Typhi, lending to its classification as the "typhoid toxin." Recently, this important virulence factor has been identified and characterized in multiple nontyphoidal *Salmonella* (NTS) serotypes as well. The significance of S-CDT in salmonellosis with regards to the: (i) distribution of S-CDT encoding genes among NTS serotypes, (ii) contributions to pathogenicity, (iii) regulation of S-CDT expression, and (iv) the public health implication of S-CDT as it relates to disease severity, are reviewed here (Figure 2.1).

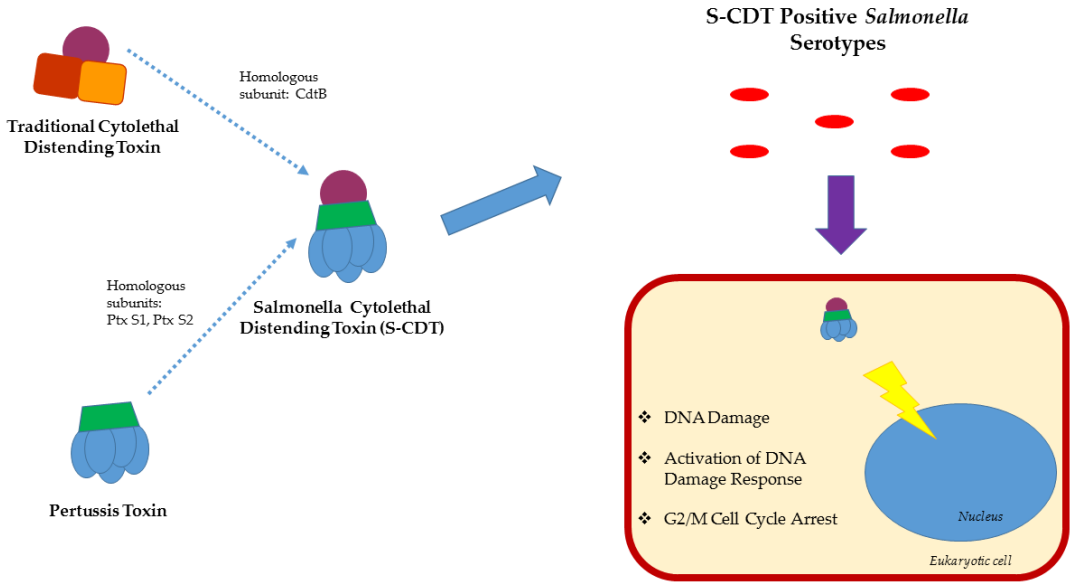

FIGURE 2.1 Overview of the structure and function of the salmonella cytolethal distending toxin (S-CDT). S-CDT combines the active subunit, CdtB, from the traditional cytolethal distending toxin, with the pertussis toxin subunits, to form a novel toxin which induces DNA damage resulting in activation of the DNA damage response, and subsequent G2/M cell cycle arrest.

INTRODUCTION

Salmonella enterica (*S. enterica*) is a Gram-negative bacterium that causes gastrointestinal illness in humans and animals. The genus *Salmonella* includes two species, *enterica* and *bongori*. Within the species *S. enterica*, there are six subspecies: *enterica* (subspecies I), *salamae* (subspecies II), *arizonae* (subspecies IIIa), *diarizonae* (subspecies IIIb), *houtenae* (subspecies IV), and *indica* (subspecies VI) [1]. Subspecies *enterica* is further categorized into 1586 serotypes (e.g., Typhimurium, Typhi, Newport, and Enteritidis), representing unique antigenic formulae of the O and H antigens [2]. For simplicity, *S. enterica* serotypes may be further categorized as “typhoidal” (*i.e.*, *S. enterica* serotype Typhi [*S.* Typhi]), “paratyphoidal”

(*i.e.*, *S. enterica* serotypes Paratyphi A, B, or C) or nontyphoidal (*i.e.*, *S. enterica* serotypes except Typhi, and Paratyphi A, B, or C) [3].

Salmonellosis, the disease resulting from a *Salmonella* infection, is primarily acquired through the consumption of contaminated food or water. In the US, foodborne salmonellosis accounts for an estimated 1.03 million cases of foodborne illness per year [4]. Internationally, nontyphoidal salmonellosis is responsible for an estimated 80.3 million illnesses and 150,000 deaths per year [5]. Importantly, some serotypes (e.g., Typhimurium, Newport, and Enteritidis) are capable of causing disease in a wide range of hosts, including humans and other mammals, birds, and reptiles, while others are host-restricted (e.g., *S.* Typhi in humans) [6,7].

Although not fully understood, *S. enterica* serotypes differ in virulence, with some serotypes being more commonly associated with invasive disease, and others causing a self-limiting gastroenteritis [6]. *S.* Typhi, the causative agent of typhoid fever, causes a severe, sometimes life-threatening illness. Serotypes Paratyphi A, B, and C cause a similar illness known as paratyphoid fever [3]. Whole genome sequence comparisons of serotype Typhi and nontyphoidal serotypes have failed to definitively account for differences in virulence [8,9]. Recently, *S.* Typhi was found to encode a variant of the cytolethal distending toxin (CDT), an important virulence factor for several other Gram-negative bacteria [10]. This novel form of CDT (hereafter referred to as "S-CDT" for Salmonella CDT) was believed to be unique to *S.* Typhi, leading to its classification as the "typhoid toxin" [8,11,12]. However, S-CDT has since been identified in at least 40 NTS serotypes [13]. Our current understanding of S-CDT with regards to its regulation, structure, function, and mechanism of action has primarily been informed by characterization of S-CDT produced by *S.* Typhi. The established genetic and pathogenic differences among *S. enterica* serotypes, particularly Typhi and nontyphoidal

serotypes, warrant further characterization of S-CDT among different NTS serotypes. This review will: (i) summarize the current understanding of the distribution, production, structure and function, and cytotoxic effects of S-CDT produced by *S. enterica* serotypes; and (ii) compare the unique features of S-CDT to the CDTs produced by other Gram-negative bacteria.

SALMONELLA ENCODES A NOVEL FORM OF CDT

CDT was first characterized in *Escherichia coli* (*E. coli*) in the late 1980s by Johnson and Lior, who noted that eukaryotic cells which were co-incubated with filtrates of overnight cultures of *E. coli* appeared distended, and arrested in the G2/M phase [14]. Subsequent analyses also identified CDT production by other Gram-negative pathogens, including *Campylobacter* spp. [15–18], *Haemophilus* spp. [19,20], *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) [21], *Helicobacter* spp. [22–26], *Shigella* spp. [27], *Yersinia* spp. [10], *Providencia alcalifaciens* [28], and *S.* Typhi [12,29]. The CDT encoded by all of these pathogens, with the exception of *S.* Typhi, exists as a tripartite AB_2 toxin encoded by the genes *cdtA, cdtB,* and *cdtC,* with the CdtB subunit serving as the active component of the toxin, and subunits CdtA and CdtC implicated in binding to host cells and subsequent intracellular trafficking [10]. S-CDT represents an important exception, as *Salmonella* strains producing S-CDT encode *cdtB,* but not *cdtA* or *cdtC* [11,12,29]. In contrast to the AB_2 configuration of CDTs encoded by other Gram-negative bacteria, S-CDT is an A_2B_5 toxin, comprised of toxin subunits: (i) CdtB (encoded by *cdtB,* cytolethal distending toxin subunit B), a nuclease subunit; (ii) PltA (encoded by *pltA*, pertussis like toxin subunit A), an ADP-ribosylating toxin subunit; and (iii) PltB (encoded by *pltB,* pertussis like toxin subunit B), serving as a pentameric ring constituting the binding subunit [11]. Subunits PltA and PltB share homology with the Ptx S1 (active) and Ptx S2 (binding) subunit, respectively, of the pertussis toxin, which ADP-ribosylates host G

proteins [11,30]. Similarly, the 3D configuration of the PltB subunit also aligns well with the binding subunit (SubB) of the *E. coli* subtilase toxin, which is a serine protease [8,31]. Recent studies have identified genes encoding S-CDT in a number of NTS serotypes as well [9,13,32–34]. To date, genes encoding S-CDT (*i.e.*, genes *pltA*, *pltB*, and *cdtB*) have been characterized in at least 40 NTS serotypes (see Table 2.1) [9,13,32]. Amino acid alignments of CdtB, PltA, and PltB from both NTS serotypes and serotype Typhi suggest that these proteins are highly conserved among *S. enterica* serotypes [34]. Genomic analyses have also detected orthologs of genes encoding S-CDT in *S. bongori* and *S. enterica* subsp. *arizonae*, although the functionality of these gene products has not been assessed [34,35]. Further DNA-based analyses will aid in the characterization and detection of genes encoding S-CDT in other NTS serotypes, and will likely expand the list of NTS serotypes known to encode S-CDT.

TABLE 2.1 Salmonella cytolethal distending toxin (S-CDT) status of select *Salmonella enterica* subspecies *enterica* serotypes.

Serotype	S-CDT Status [1]	References
9,12:I,v:-	−	[9]
Agbeni	+	[13]
Agona	−	[13]
Anatum	−	[13]
Arechavaleta	+	[13]
Bareilly	−	[13]
Barranquilla	+	[13]
Berta	−	[13]
Braenderup	−	[13]
Brandenburg	+	[13]
Bredeney	+	[9]
Choleraesuis	−	[9,13]
Corvallis	+	[13]
Cotham	+	[13]
Cubana	+	[13]
Dublin	−	[9,13]
Enteritidis	−	[9,13]
Freetown	+	[13]
Gaminara	+	[13]
Georgia	+	[13]
Give	+	[13]
Glostrup	+	[13]
Hadar	−	[9,13]
Hartford	−	[13]
Heidelberg	−	[9,13]
4,[5],12:i:-	−	[13]
Indiana	+	[13]
Infantis	−	[13]
Inverness	+	[13]
Javiana	+	[13,32]
Johannesburg	+	[13]
Kiambu	+	[13]
Kintambo	+	[13]
Kisarawe	+	[13]
Luciana	+	[13]
Miami	+	[13]
Minnesota	+	[13]
Mississippi	±	[13]
Montevideo	+	[9,13,36]
Muenchen	−	[13]
Muenster	+	[13]
Newport	−	[9,13]
Oranienburg	+	[13]
Overschie	+	[13]
Panama	+	[13]
Paratyphi A	+	[13]
Pomona	+	[13]
Poona	+	[13]
Reading	+	[13]
Rubislaw	+	[13]
Sandiego	+	[13]
Schwarzengrund	+	[9,13]
Telelkebir	+	[13]
Thompson	−	[13]
Typhi	+	[12,29]
Typhimurium	−	[9,13]
Urbana	+	[13]
Virchow	−	[9,13]
Wandsworth	+	[13]

[1] Status based on the presence of all S-CDT encoding genes (*pltA, pltB,* and *cdtB*) as determined by PCR-based amplification; ‘‘+’’ denotes all genes are present; ‘‘−’’ denotes one or more genes were not detected; ‘‘±’’ denotes some isolates within the serotype are positive, but others are negative (unpublished data).

REGULATION OF S-CDT EXPRESSION

Several reports have confirmed that S-CDT expression is restricted to intracellular *S.* Typhi residing within the salmonella containing vacuole (SCV) [11,12]. Importantly, this is in contrast to CDT production by other Gram-negative bacteria, for which the toxin is routinely detected in cell-free supernatants of CDT positive strains cultivated in standard laboratory media [14,19,37,38]. The intracellular requirement for S-CDT production has not yet been confirmed for NTS expressing CDT.

The requirement of bacterial internalization for S-CDT expression by *S.* Typhi has been confirmed at both the transcriptional and translational levels. Haghjoo and Galán used a luciferase reporter strain to establish that *cdtB* is not expressed by *S.* Typhi grown in lysogeny broth (LB), and that transcription was only activated when *S.* Typhi was allowed to infect eukaryotic cells [12]. Furthermore, epithelial cells infected with an invasion-deficient mutant of *S.* Typhi did not have the characteristic distended phenotype, nor did they arrest in the G2/M phase, suggesting that invasion, and not just adhesion, is required for S-CDT production by *S.* Typhi [12]. However, transcription of *pltA* and *pltB* can be detected when *S.* Typhi is grown in standard LB media, although at very low quantities [11]. This is likely due to the organization of the CdtB-islet into two distinct operons encoding the toxin subunits [11]. Taken together, the fact that *pltA* and *pltB* are located in an operon separate from *cdtB,* and that transcription of *pltA* and *pltB,* but not *cdtB,* may occur in standard culturing medium, suggests that *pltA* and *pltB* may be regulated separately of *cdtB.*

A transposon mutagenesis screen identified IgeR, a transcriptional regulator belonging to the DeoR family of transcriptional regulators, as a repressor of *cdtB* transcription in *S.* Typhi [39]. *In vitro* analyses determined that IgeR is able to bind to the *cdtB* promoter, and effectively suppress *cdtB* expression [39]. Likewise, deletion of *igeR* was sufficient to de-repress *cdtB* expression in LB media, a normally non-permissive environment for *cdtB* expression by *S.* Typhi [39]. IgeR also plays a role in the regulation of other genes involved in virulence, including SPI-1 encoded type three secretion system (TTSS) components, flagellar proteins, and SPI-1 TTSS effector proteins, as deletion of *igeR* resulted in decreased expression of these genes [39]. IgeR is conserved among *S. enterica* subsp. *enterica* serotypes, and hence could also control transcription for S-CDT production in NTS [39]. In addition, *cdtB* transcription was found to be activated concurrently with *parE* and *mntR*, but repressed with transcription of *potG* and *tldD,* although the exact mechanisms regarding their regulation are currently unknown [39]. In support of IgeR-mediated repression of *cdtB* transcription, plasmid-based expression of *cdtB* under control of its native promoter, in a heterologous bacterial host (*i.e.*, *S.* Typhimurium), was found to be sufficient for constitutive expression of the CdtB-islet under conditions that are normally non-permissive for wild type strains of *S.* Typhi [39,40]. Another study suggested that the two component PhoQ-PhoP regulatory system may also play a role in *cdtB* expression in *S.* Typhi, as increased levels of *cdtB* mRNA transcripts and CdtB were detected when *Salmonella* cells were subjected to PhoP-inducing conditions [41]. As the CdtB-islet constitutes two operons, and expression of the *pltAB* operon may be detected when S-CDT positive strains are cultured under conditions that are normally non-permissive for expression of the operon containing *cdtB*, it is unclear whether IgeR also regulates transcription of the *pltAB* operon. Taken together, these results suggest that regulation of S-CDT expression in *S.* Typhi at the transcriptional level

involves multiple regulatory components, which are likely also involved in the regulation of invasion-associated genes.

Two additional genes, *sty1887* and *sty1889* within the CdtB-islet, are implicated in S-CDT gene regulation as well (see Fig 2.2) [42]. Deletion of *sty1889* (renamed *ttsA*), but not *sty1887*, abrogated secretion of S-CDT in a *S.* Typhi strain, and prevented subsequent intoxication of epithelial cells [42]. *In silico* analysis implicates that *ttsA* encodes a *N*-acetyl-β-D-muramidase, with homology to a bacteriophage muramidase [42]. Similar to CdtB, TtsA is not detected in standard LB culturing medium, and is only detected when *S.* Typhi infects a host cell [42]. Further analyses determined that the TtsA peptidoglycan binding domain is required for S-CDT secretion [42]. Currently, all S-CDT regulatory analyses have been performed in serotype Typhi. Due to the marked differences between Typhi and NTS serotypes, it will be important to characterize the regulation and expression of S-CDT in NTS.

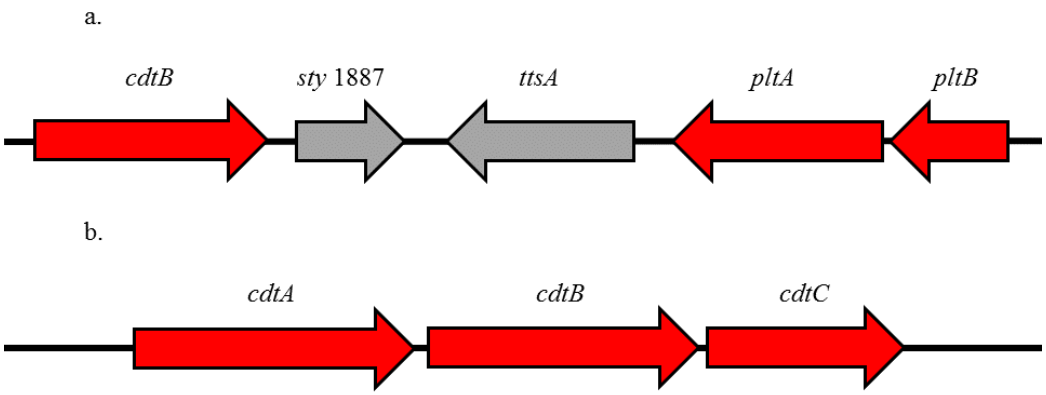

FIGURE 2.2 Comparison of the CdtB-islet encoded by *Salmonella enterica* serotypes and all other Gram-negative species producing a cytolethal distending toxin (CDT). (**a**) The *Salmonella* CDT (S-CDT) is comprised of subunits PltA, PltB, and CdtB encoded by *pltA*, *pltB*, and *cdtB*, respectively. The CdtB-islet in *Salmonella* also encodes two genes (*sty1887* and *ttsA*) which are implicated in toxin secretion but are not subunits of S-CDT [39,42]. (**b**) Genes *cdtA*, *cdtB*, and *cdtC* encode the CDT for *Aggregatibacter actinomycetemcomitans*, *Campylobacter* spp., *Escherichia coli*, *Haemophilus* spp., *Helicobacter* spp., *Providencia alcalifaciens*, *Shigella* spp., and *Yersinia* spp. Genes colored red compose the CDT and S-CDT; genes shown in gray are present in the CdtB-islet of *Salmonella*, but do not encode subunits of the S-CDT.

ARTA AND ARTB AND THEIR RELATIONSHIP TO S-CDT

Homologs to genes encoding the PltA and PltB subunits of S-CDT have also been detected in a number of NTS serotypes [13]. First identified in *S. enterica* serotype Typhimurium strain DT104, genes encoding an ADP-ribosyl transferase toxin homolog (*artA* and *artB*) have been characterized on a putative prophage in serotype Typhimurium, as well as in other NTS serotypes [13,43]. The protein encoded by *artA* is homologous to both the pertussis-like toxin subunit in *S.* Typhi (encoded by *pltA*) and the S1 subunit of the pertussis toxin (encoded by *ptxA*), with the predicted amino acid products sharing 59% and 33% amino acid identity, respectively [43]. A second subunit, ArtB, has homology to the amino acid product encoded by *pltB* (30% amino acid identity), as well as the S2 and S3 subunits (30.7% amino acid identity) of the Ptx binding component of the pertussis toxin [43]. Genome alignments have detected *artA* and *artB* in the majority of NTS serotypes encoding the CdtB-islet [13]. For these serotypes, the location of *artA* and *artB* was inconsistent, providing support for the genes being encoded on a prophage [13]. Despite the seemingly widespread distribution of *artA* and *artB*, the function and potential contributions of *artA* and *artB* gene products to virulence remain unknown. Likewise, it is unclear if *artA* and *artB* are expressed concurrently with genes in the CdtB-islet. While all three subunits of S-CDT are required for full activity, some studies have shown that deletion mutants of *pltB* retain some residual cytotoxic activity [32]. Therefore, it would be interesting to examine whether ArtA or ArtB, or both, can potentially substitute for PltA or PltB. A recent study analyzing the 3D crystal structure of S-CDT from *S.* Typhi predicted that three cysteine residues in the PltA subunit serve as the physical link between CdtB and PltA [8]. In contrast, the ArtA subunit only contains two such cysteine residues that could interact with CdtB, and therefore CdtB is predicted to be preferentially bound by PltA rather than ArtA [8]. ArtA and

ArtB appear to be more widespread among NTS, as they are also present in strains that do not encode S-CDT [43]. However, the activity and effects on host cellular processes resulting from the "ArtAB toxin" remain uncharacterized.

STRUCTURE AND FUNCTION OF S-CDT

S-CDT is arranged in an A_2B_5 configuration (see Fig 2.3) [8]. In its final quaternary form the toxin exists as a pyramid-shaped structure that is ~90 Å tall with a maximum width of ~60 Å (at the base) [8]. Five PltB subunits (encoded by a single copy of *pltB*) form a pentameric ring at the base of the toxin [8]. The pentameric ring is covalently linked to PltA at its carboxy terminus, which inserts into the hydrophobic alpha-helical ring of the PltB pentamer [8]. A disulfide linkage between PltA Cys214 and CdtB Cys269 anchors CdtB at the most distal location from S-CDT's pentameric base (Fig 2.3) [8]. Therefore, CdtB does not physically interact with the PltB subunits [8]. Structurally, the disulfide bonds and catalytic residues of both the pertussis toxin S1 (Glu129) and the PltA subunit of S-CDT (Glu 133) overlap in the 3D configuration, suggesting that reduction of the disulfide bonds would be necessary for activation of the ADP-ribosylating function of PltA, as is the case for the pertussis toxin [8]. Alignment of the 3D protein structures of the S-CDT subunits PltA, PltB, and CdtB with their respective homologous protein subunits (*i.e.*, Ptx S1 with PltA, Ptx S2 or SubB with PltB, and CdtB from *S.* Typhi with CdtB from *Haemophilus ducreyi* (*H. ducreyi*) yielded low root-mean-square-deviations [8]. This further supports the hypothesis that the subunits PltA and PltB share homology to subunits of the pertussis and subtilase toxins, respectively, and also share a common structure and function (Fig 2.3) [8].

The translated product of *pltB*, encoding the pentameric B-subunit of S-CDT, is 137 amino acids in length, composed of a 23 aa secretion signal peptide and a 114 aa chain [44].

Interestingly, the amino acid sequence of PltB, as well as the 3D configuration, aligns well with the SubB subunit of the subtilase toxin encoded by *E. coli* [8,31]. Analogous to SubB, PltB is implicated in binding to host cells [8,31]. Chromatography-based interaction studies have identified several possible host cell receptors for the PltB subunit of S-CDT, namely podocalyxin-like protein 1 (PODXL), but also a variety of sugar moieties on glycoproteins and glycoplipids, including sialylated glycans [8]. Given S-CDT's ability to intoxicate a wide variety of cell types, it is likely that PltB is able to bind to a variety of host cell structures, namely glycans [8]. Similarly, the SubB subunit of the subtilase toxin preferentially recognizes and binds to sialylated glycoproteins [31]. There are conflicting reports regarding the requirement of PltB for cytotoxicity [11,32]. A Δ*pltB* mutant of *S.* Typhi failed to induce a G2/M cell cycle arrest in a cell culture model, suggesting that PltB plays a critical role in toxin trafficking [8,12]. However, HeLa cells infected with a Δ*pltB* mutant of *S. enterica* serotype Javiana (*S.* Javiana) showed evidence of a G2/M phase arrest, consistent with S-CDT [32]. Purified PltB has been shown to up-regulate chemokine and cytokine production in a cell culture model as well, suggesting that its role in virulence may not be limited to just ensuring delivery of CdtB to host cells [45].

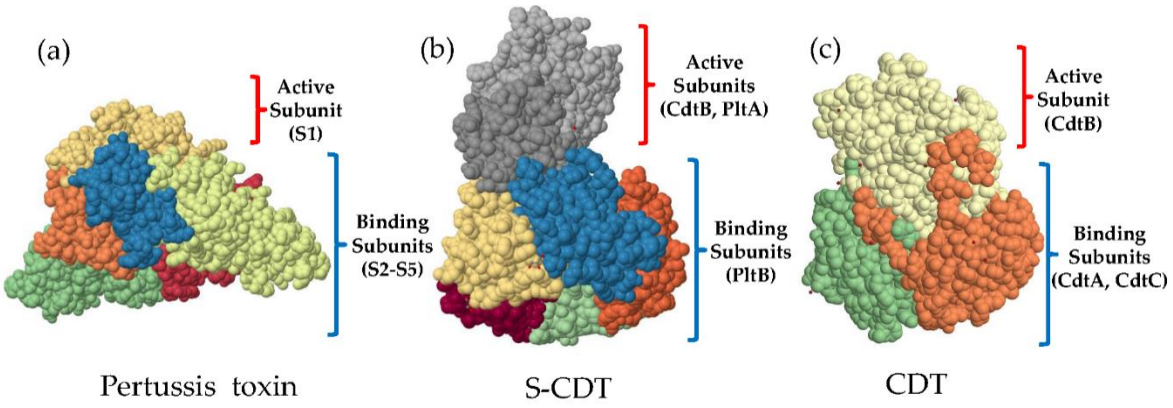

FIGURE 2.3 Space-fill models of the 3D structure of the: (**a**) pertussis toxin; (**b**) cytolethal distending toxin (CDT) from *Salmonella enterica* serotype Typhi; and (**c**) CDT from *Haemophilus ducreyi.* The unique A_2B_5 structure of the Salmonella cytolethal distending toxin (S-CDT) combines active subunits CdtB from the CDT produced by other Gram-negative species, and the ADP-ribosyltransferase toxin subunit of the pertussis toxin. The binding subunit of S-CDT is arranged as a pentameric ring, similar to the binding portion of the pertussis toxin. Protein databank accession numbers: Pertussis toxin (1PRT) [46], S-CDT (4K6L) [8], and CDT (1SR4) [47].

PltA, one of S-CDT's two active subunits, is a functional ADP-ribosylating subunit with homology to the active subunit of the pertussis toxin [8,11,13]. The 27.1 kDa PltA subunit consists of 242 aa, comprising both a signal sequence peptide of 18 aa residues and a 224 aa chain [48]. The functionality of PltA as an ADP-ribosyltransferase has been confirmed in *S.* Typhi, however the host protein target(s) remain(s) unknown [11]. In *Bordetella pertussis*, the causative agent of whooping cough, the pertussis toxin plays a critical role in modulating the host immune response by ADP-ribosylating host G proteins, and subsequently disrupting G protein signaling pathways [30,49,50]. Importantly, eukaryotic cells infected with *S.* Typhi Δ*pltA* mutants do not have the characteristic distended phenotype [11,32]. However, substitution of PltA with a catalytically inactive variant $PltA^{E133A}$, restored S-CDT-induced cytotoxicity, suggesting that despite PltA's functioning as an active ADP-ribosylating toxin, its role in S-CDT-mediated cytotoxicity is most likely related to entry and trafficking of S-CDT in intoxicated eukaryotic cells, as the subunits CdtB and PltB do not physically interact [8,11].

Further elucidation of the molecular targets of PltA-mediated ribosylation will be necessary to fully understand its role as a virulence factor, and furthermore, its role in S-CDT-mediated cytotoxicity. While PltA does not appear to play an important role in the DNA damaging activity of the CdtB subunit, it will be important to identify the molecular targets of the ADP-ribosyl transferase in order to elucidate its potential contributions to the outcome of an infection with a S-CDT positive strain.

The cytotoxic effects associated with S-CDT intoxication are primarily attributable to the CdtB subunit. The CdtB subunit has limited amino acid sequence homology to mammalian DNase I, and is thought to cleave host DNA, thereby triggering activation of the host cell's DNA damage response (DDR), resulting in the distended morphology and G2/M cell cycle arrest [10,51]. The CdtB subunit may also act as a phosphatase, as the CDT produced by *A. actinomycetemcomitans* has demonstrated PI-3,4,5-triphosphate phosphatase activity, although phosphatase activity has yet to be confirmed for S-CDT [52]. The CdtB subunit is highly conserved among CDT positive *Salmonella* [34]. The CdtB subunit has a mass of 29.6 kDa, and is 269 aa in length, comprising a 22 aa signal peptide and 247 aa chain [48]. In agreement with characterization of the CDT produced by other Gram-negative bacteria, CdtB is necessary for the distended phenotype of infected cells, as deletion of *cdtB* in *S.* Typhi and NTS strains results in a loss of the ability to elicit a G2/M phase arrest in eukaryotic cells [11,12,32,34,51]. Transfection of a Cos-2 cell line with plasmid-encoded *S.* Typhi *cdtB* was sufficient for cytotoxicity, further supporting CdtB as the active component of S-CDT [12]. Despite CdtB's confirmed activity, it is still unclear if CdtB preferentially targets certain DNA motifs, and how many single strand breaks (SSB) and/or double strand breaks (DSB) it may introduce into any given strand of DNA.

MECHANISM OF ACTION

The delivery and trafficking of S-CDT differs from that of the CDT produced by other Gram-negative bacteria. The key differences distinguishing S-CDT trafficking and activation from CDTs produced by other Gram-negative bacteria include: (i) S-CDT is only produced when *Salmonella* cells are residing within a host eukaryotic cell; (ii) S-CDT must be exported out of the SCV and subsequently out of the host cell, after which the exported S-CDT may either re-enter the cell or intoxicate a nearby cell; (iii) S-CDT's unique A_2B_5 structure (compared to the AB_2 configuration of other CDTs) requires a reducing atmosphere to dissociate the PltA and CdtB subunits; and (iv) the host cell receptors for S-CDT differ as a reflection of its use of PltB rather than CdtA and CdtC subunits for binding to host cells.

S-CDT Uses Multiple Host Cell Receptors Enabling it to Intoxicate a Wide Variety of Cell Types. Collectively, S-CDT and other CDTs are able to intoxicate a wide variety of host cells [8,51]. Despite this, several reports suggest that CDT binding and intracellular trafficking within host cells is species specific, with different receptors and intracellular trafficking mechanisms being utilized depending on the bacterial species producing the CDT [51,53–56]. A recent study suggested that S-CDT binds to a variety of host receptors, including PODXL, and CD45 on B and T cells [8].

Song *et al.* noted that sugar moieties of primarily glycoproteins, but also glycolipids, are the primary target for S-CDT binding [8]. S-CDT preferentially binds α(2-3)-linked *N*-acetylneuraminic acid [8]. In comparison, the B subunit of the subtilase toxin (which has homology to the B subunit of S-CDT) binds preferentially to α(2-3)-linked *N*-glycolylneuraminic acid terminating glycans, but also α(2-3)-linked *N*-acetylneuraminic acid glycans [31]. Similar to

the pertussis toxin S2 binding subunit, S-CDT also demonstrates some affinity for terminal sialic acid moieties [8,30].

The CDTs produced by other Gram-negative bacteria may also use N-linked carbohydrate structures as receptors. Initially, *E. coli* CDT was characterized as binding to N-linked carbohydrate moieties of glycoproteins, while the CDT produced by *A. actinomycetemcomitans* preferentially uses the ganglioside GM3 as the cell receptor [57,58]. Eshraghi *et al.* noted that the CDTs produced by *E. coli, H. ducreyi, Campylobacter jejuni*, and *A. actinomycetemcomitans* were affected differently by host cell N-linked glycosylation, cholesterol levels, and deficiencies in sialic acid, galactose and glycolipids, therefore suggesting that the CDT mode of entry is dependent on the bacterial species producing the CDT [55]. In summary, like other CDTs, it appears that S-CDT does not utilize a single receptor. Rather, S-CDT can utilize multiple different receptors, perhaps explaining why S-CDT is capable of intoxicating a number of different cell types [8,34,40]. It has been suggested that the variability in host cell receptors utilized by CDTs from different bacterial species, may partially explain why certain CDT-producing pathogens preferentially inhabit and colonize particular regions of the host [10,51,55,59].

Entry and Trafficking of S-CDT. Intracellular trafficking and subsequent targeting of CdtB to the nucleus occurs via different mechanisms, depending on the bacterial species producing the CDT [10,56]. In the case of S-CDT, following its production by Salmonella residing within the SCV, it appears that S-CDT must first be exported out of the infected host cell, before being endocytosed by either the eukaryotic host cell from which it was produced, or by another cell [11]. The most convincing evidence for this hypothesis was generated by Spanò et al., who showed that addition of a toxin-neutralizing antibody prevented intoxication of epithelial cells

that were infected with S. Typhi cells actively producing S-CDT [11]. The secretion of S-CDT out of the SCV, and subsequently out of the host cell, requires the production of outer membrane vesicles (OMV), which "bud" off of the SCV, and are trafficked by host kinesin along microtubules to the plasma membrane [40].

Re-entry of S-CDT into a eukaryotic cell infected with *Salmonella* (autocrine pathway), or entry into an uninfected cell (paracrine pathway), occurs via endocytosis (See Fig 2.4). Similarly, the pertussis toxin, subtilase toxin, and CDTs produced by other Gram-negative bacteria, also utilize endocytosis for toxin entry [30,31,51]. While the requirement of clathrin in the endocytosis of S-CDT is currently unknown, endocytosis of other CDTs may occur via clathrin-dependent or clathrin-independent mechanisms, while endocytosis of the subtilase toxin is clathrin-dependent [31,51,53,60]. Following endocytosis, S-CDT is predicted to follow retrograde trafficking through the Golgi complex and endoplasmic reticulum [40]. For *H. ducreyi*, endosomal trafficking transports the CdtB and CdtC subunits retrograde to the trans-Golgi network, and then subsequently through the Golgi complex via COPI vesicles, as evidenced by sulfation (a Golgi-specific activity) of CdtB, and the absence of a distended phenotype when intoxicated cells were treated with Brefeldin A, which inhibits the formation of COPI vesicles [59,61]. Subsequent transportation of S-CDT across the nuclear membrane, and into the nucleus of the host cell where it elicits SSB and/or DSB, is currently uncharacterized. It is still unclear how CdtB dissociates from the other components of S-CDT, and at which stage this occurs [8]. Presumably, the disulfide bond between PltA Cys214 and CdtB Cys269, is reduced by host cell reductases [8]. By comparison, the pertussis toxin active component is dissociated, and therefore activated, in the ER prior to being released into the cytosol where it ADP-ribosylates G proteins [30]. It is possible that S-CDT components PltA and CdtB separate

in the ER as well. Following exit of the ER, the CdtB subunit must cross the nuclear membrane, and enter the host cell nucleus in order to induce DNA damage.

The current understanding of the intracellular trafficking of S-CDT is largely based on the intracellular trafficking of related toxins, namely the CDTs produced by other Gram-negative bacteria, and the pertussis and subtilase toxins. However, some studies have demonstrated that CDTs may utilize different intracellular trafficking mechanisms, requiring different components of the host cell for trafficking to the nucleus [56,62]. For example, treatment of HeLa cells with chemical agents blocking endosomal acidification (e.g., bafilomycin A1 or ammonium chloride) prevented *H. ducreyi* CdtB transportation to the nucleus, but not *E. coli* CdtB trafficking to the nucleus [56]. These results suggest that *E.coli* and *H. ducreyi* CDTs utilize different intracellular trafficking mechanisms to elicit their cytotoxic effects [56].

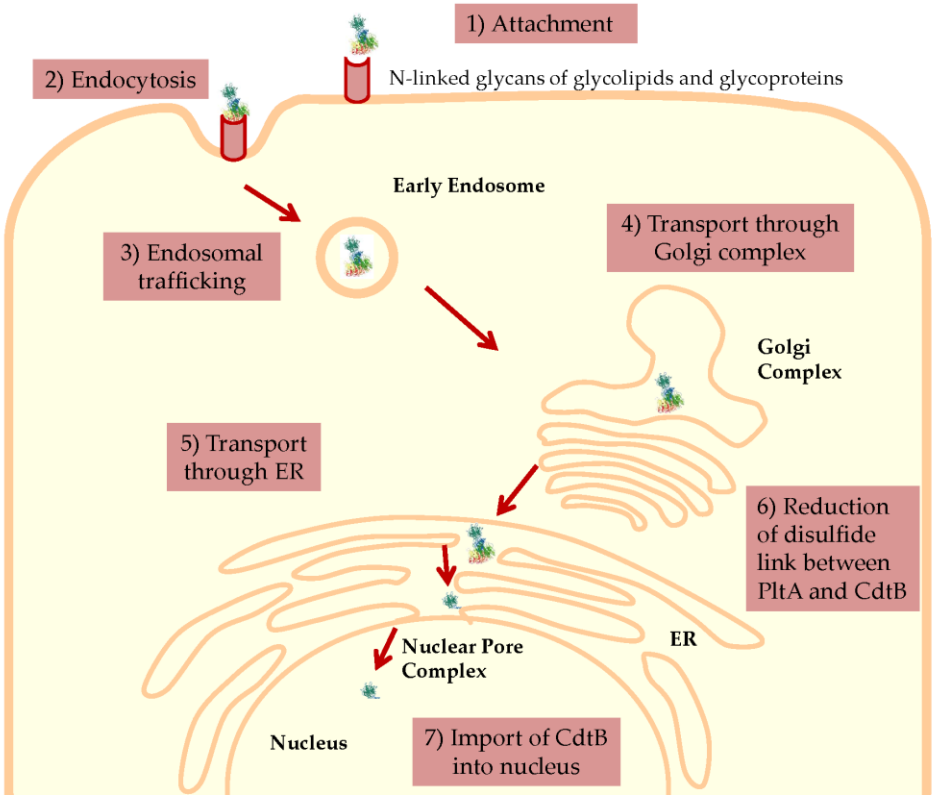

FIGURE 2.4 Proposed model for the entry and intracellular trafficking of Salmonella cytolethal distending toxin (S-CDT). (1) The PltB subunits of S-CDT bind to sugar moieties on glycoproteins and glycolipids on the host cell surface [8]. (2) S-CDT is internalized by endocytosis and is (3) trafficked in endosomes, which deliver the S-CDT to the Golgi complex [11]. Subsequently, S-CDT is (4) transported retrograde through the Golgi complex, likely mediated by COPI vesicles, and then (5) through the endoplasmic reticulum [53]. (6) It is hypothesized that in the ER, host reductases reduce the disulfide bonds covalently linking the PltA and CdtB subunits, releasing CdtB from the holotoxin [8]. (7) The CdtB subunit is imported into the nucleus, likely by passing through the nuclear pore complex, as is done for other CDTs [53]. Once in the nucleus, CdtB acts as a nuclease and cleaves host DNA to activate the host cell's DNA damage response [40]. Protein databank entry for S-CDT (4K6L) [8]. Cell adapted from [63].

Importantly, S-CDT has multiple structural differences in comparison to the CDTs produced by other Gram-negative bacteria, namely, the absence of CdtA and CdtC subunits, and the presence of subunits PltA and PltB subunits. Therefore, further research will be necessary to confirm the exact trafficking mechanisms of S-CDT produced by Typhi and nontyphoidal serotypes.

S-CDT'S ROLE IN VIRULENCE

Characterizations of the deleterious effects associated with S-CDT intoxication at both the cellular and organismal levels have provided key insights into the contributions of S-CDT to disease.

DNA Damage and Induction of the DNA Damage Response. The hallmark of CDT-intoxication is the production of SSB and/or DSB, resulting in activation of the intoxicated host cell's DDR, and subsequent G2/M phase arrest and cellular distention (see Table 2.2) [10,51,64,65]. This is also true of S-CDT, and has been confirmed for S-CDT produced by both Typhi and NTS serotypes [8,11,12,32–34,42]. Interestingly, CDT-mediated DNA damage preferentially results in G2/M phase arrest [10,16,17,19,21–26,38,51,65]. However, it should be noted that cells arrested in the G2/M phase may have sustained damage prior to entering the G2 phase [51,64,66]. The majority of studies reporting G2/M phase arrest in CDT-intoxicated cell populations used DNA content to attribute cells to a defined growth phase [10,12,51,59]. However, the quantification of DNA within a given cell would not distinguish damage that occurred and was detected prior to G2 phase, versus damage occurring in G2 [51,66]. In support of this, Fedor et al. determined that for HeLa cells intoxicated with low doses of E. coli CDT, SSB were converted to DSB in the S-phase [64]. Therefore, it is likely that CDT and S-CDT DNase activity induces DNA damage regardless of the eukaryotic cell cycle phase, but the actual cell arrest is evident in the G2/M phase transition.

TABLE 2.2. Pathogenic outcomes attributed to intoxication with Salmonella cytolethal distending toxin and other Gram-negative bacteria producing cytolethal distending toxins.

Pathogenic Outcome of CDT-Mediated Intoxication	**Bacterial Species** [1]	**References**
Cellular Outcomes		
G2/M Phase arrest	*A. actinomycetemcomitans* *C. jejuni* *E. coli* *Haemophilus* spp. *Helicobacter* spp. *P. alcalifaciens* *Shigella* spp. *Salmonella* (Typhi and NTS)	[12,14,15,17,19,22, 24,27,28,34,67]
Activation of host cell DNA damage response	*A. actinomycetemcomitans* *C. jejuni* *E. coli* *Haemophilus* spp. *H. ducreyi* *H. hepaticus* *P. alcalifaciens* *S.* Typhi	[26,28,40,64,68–71]
Induction of autophagy	NTS	[33]
Induction of apoptosis	*A. actinomycetemcomitans* *C. jejuni* *E. coli* *H. ducreyi* *Helicobacter* spp. *P. alcalifaciens* NTS	[33,72–78]
Host Outcomes		
Tumorigenesis and neoplastic lesions	*H. cinaedi* *H. hepaticus* *H. ducreyi*	[79–82]
Typhoid-like illness	*S.* Typhi	[8]
Chronic infection	*H. hepaticus*	[83]

[1] Pathogenic outcome reported for CDT produced by given bacterial species. NTS refers to "nontyphoidal *Salmonella*".

Following detection of DNA damage, the host cell's DDR is activated. While the activation of DDR proteins has not been reported for S-CDT, studies of CDT-mediated intoxication for

other Gram-negative bacteria have confirmed the activation of the MRN complex (a complex of Mre11, Rad50 and Nbs1) in the ataxia telangiectasia mutated (ATM) dependent DNA damage signaling pathway, as well as phosphorylation of the C-terminal serine 139 of histone H2AX (called γH2AX), which is commonly associated with DSBs [40,64,67,69,77,84,85]. In addition, single cell electrophoresis of CDT-intoxicated cells (also referred to as the "comet assay") has demonstrated DNA fragmentation, indicating that CdtB is capable of inducing multiple lesions in the host DNA [64,85,86]. However, Fahrer *et al.* also suggested that CDT is capable of activating the ataxia telangiectasia and Rad3 related (ATR) mediated DDR signaling pathway, but at a delayed rate compared to the ATM-dependent signaling pathway [85]. Finally, the DNA damage induced by S-CDT and the CDTs of other Gram-negative bacteria causes nuclear enlargement and a distended morphology among intoxicated cells [10–12,14,51].

Apoptosis of Immune Cells and Host Immune Suppression. Apoptosis resulting from CDT-intoxication has been demonstrated for a wide range of host cell types, including immune and non-immune cell types [33,73,87–90]. Williams *et al.* demonstrated that S-CDT produced by *S.* Javiana induced apoptosis in J774A.1 macrophage cells, which also had a significant increase in expression of the pro-apoptotic *Bax* gene compared to J774.A1 cells infected with a *S.* Javiana Δ*cdtB* isogenic mutant [33]. Currently, it appears that CDT-mediated induction of apoptosis occurs primarily via the intrinsic pathway, through increased expression of Bax and activation of caspase 9 and subsequently caspase 3 [51,77].

Tumorigenesis and Carcinogenic Potential. Chronic exposure to CDT has been investigated for several Gram-negative bacterial species. Despite similar hepatic colonization levels, mice infected with CDT positive *Helicobacter hepaticus* (*H. hepaticus*) developed hepatic dysplasic nodules, while mice infected with a CDT-null mutant did not [82]. Similarly, chronic

intoxication with purified *H. hepaticus* or *H. ducrecyi* CDT was associated with malignant transformations in a cell culture model [79]. Chronic inflammation is an important predisposition for cancer development [80,91–93]. In multiple cell culture models, administration of purified PltB (called ArtB in the study) of S. Typhi elicited expression of pro-inflammatory cytokines, possibly suggesting a role for S-CDT in the induction of inflammation [45]. Chronic infection with *S.* Typhi is significantly associated with gall bladder cancer, although the contribution to, or requirement for, S-CDT production has not yet been established [94–96]. Together, these studies implicate a potential role for CDT and S-CDT in tumorigenesis and carcinogenesis.

One of the major limitations of studying the outcomes of chronic infection with *S.* Typhi, and therefore the potential of S-CDT in tumorigenesis or carcinogenesis, has been the lack of a suitable animal model. Recently, a humanized mouse model for *S.* Typhi infection was developed [97]. Investigations into the cellular and organismal outcomes of infection with chronic exposure to S-CDT will provide important information regarding the potential for tumorigenesis or carcinogenesis associated with salmonellosis involving S-CDT positive serotypes.

Administration of S-CDT May Recapitulate Symptoms of Typhoid Fever. Injection of purified S-CDT recapitulated symptoms associated with the acute phase of typhoid fever, for a mouse model of infection [8]. Following systemic administration, mice injected with active S-CDT lost significantly more weight compared to control mice [8]. Furthermore, mice intoxicated with the wild type S-CDT showed a marked decrease in neutrophil counts, which is characteristic of typhoid fever in humans [8]. However, infection with NTS serotypes encoding S-CDT does not result in a typhoid-like illness [6,98–100]. While S-CDT may indeed contribute to typhoid fever, the widespread distribution of S-CDT among NTS, along with the marked difference in virulence

between NTS serotypes and *S.* Typhi, suggest that the typhoid toxin may not solely responsible for, but may contribute to, typhoid fever. Alternatively, the discrepancy in disease severity between infections with *S.* Typhi and NTS serotypes producing an S-CDT, could reflect differences in expression of S-CDT, as alignments of toxin-encoding gene components from NTS and *S.* Typhi suggest that *cdtB*, *pltA*, and *pltB* are highly conserved [34]. Use of the recently developed humanized mouse model may provide an opportunity to further define the contribution(s) of S-CDT to human typhoid fever [97]. More specifically, it would be interesting to establish if S-CDT contributes to immune cell depletion, and if S-CDT enhances the ability of *S.* Typhi to establish a chronic infection, as has been proposed by other groups studying colonization and persistence of other CDT-producing pathogens [83].

Persistence and Chronic Infection. Approximately 2–5% of *S.* Typhi infections result in chronic infection [101,102]. For *H. hepaticus*, CDT is required for colonization in a host model of infection [83]. It is possible that the cell cycle arrest and immune suppression associated with S-CDT intoxication may play an important role in the colonization and development of a chronic infection with *S.* Typhi as well.

DISCUSSION AND FUTURE DIRECTIONS

Overall, CDT has been implicated as an important virulence factor among Gram-negative bacteria, having been associated with the bacteria's ability to colonize, survive, and persist within the host. Still, few studies have examined these effects in regards to the S-CDT produced by select *Salmonella* serotypes. Further characterization of S-CDT regulation, production, and mechanism of action will provide important information regarding the production of the toxin during different intra- and extracellular stages of infection. Furthermore, the true benefits of S-CDT to *Salmonella* during the course of an infection remain unclear. There is a clear difference

in the severity of salmonellosis among NTS serotypes, with some serotypes being more frequently associated with invasive disease resulting in infections requiring hospitalization [6]. Could S-CDT play an important role in disease outcome? The long-term sequelae associated with salmonellosis are well established, yet the mechanisms by which these sequelae arise are poorly understood. For example, the well-established association between gall bladder cancer and chronic infection with *S.* Typhi may be attributable to chronic exposure to S-CDT [94–96]. Chronic infection with NTS is less studied, although some reports suggest that NTS may induce chronic infections in humans and in animals [103,104]. Genotoxin production by other pathogens has also been implicated in carcinogenesis in the host [73,105,106]. Further elucidation of the true long-term sequelae associated with S-CDT-mediated intoxication will provide valuable information, which may partially explain the observed differences in virulence among the NTS serotypes.

Select pathogens are differentiated based on their possessing certain virulence factors. Shiga toxin producing *E. coli* (STEC) are characterized based on the presence of stx_1 and stx_2 genes encoding shiga toxins 1 and 2, respectively [107]. In STEC infections, appropriate treatment is guided by rapid detection of the *stx* genes, as antibiotic treatment is associated with a significantly higher incidence of hemolytic uremic syndrome, and is therefore discouraged [108]. S-CDT status could influence treatment regimens, and could also serve as an epidemiological tool for comparing similar strains implicated with a common food vehicle, as is done with the *stx* genes in *E. coli* [107].

Further characterization of S-CDT has the potential to identify novel rapid detection methods for S-CDT-producing *Salmonella* in clinical settings. Characterization of this bacterial toxin may also inform the development of novel diagnostic, treatment, and prevention strategies

for salmonellosis, as demonstrated previously for a variety of diseases including botulism, *Clostridium difficile* infection, and HUS resulting from infection with shiga toxin producing *E. coli* [109–111].

CONCLUSIONS

Overall, the implications of S-CDT in the context of salmonellosis present a unique and intriguing challenge. Multiple CDT-producing pathogens have been linked to an increased incidence of cancer among chronically infected individuals [80,95,96]. The public health implications of S-CDT production by NTS should be considered, as the recent discovery of the widespread nature of the toxin among NTS suggests that, at least in the US, many individuals may be exposed to S-CDT. Future investigations relating S-CDT's role in pathogenesis, as well as implications for the long-term sequelae attributable to S-CDT-mediated intoxication will be beneficial in assessing the contributions of S-CDT to salmonellosis in both humans and animals.

REFERENCES

1. Brenner, F.; Villar, R.; Angulo, F.; Tauxe, R.; Swaminathan, B. *Salmonella* nomenclature. *J. Clin. Microbiol.* **2000**, *38*, 2465–2467.
2. Issenhuth-Jeanjean, S.; Roggentin, P.; Mikoleit, M.; Guibourdenche, M.; de Pinna, E.; Nair, S.; Fields, P.I.; Weill, F.-X. Supplement 2008–2010 (no. 48) to the White–Kauffmann–Le Minor scheme. *Res. Microbiol.* **2014**, *165*, 526–530.
3. Crump, J.A.; Mintz, E.D. Global trends in typhoid and paratyphoid fever. *Clin. Infect. Dis.* **2010**, *50*, 241–246.
4. Scallan, E.; Hoekstra, R.M.; Angulo, F.J.; Tauxe, R.V.; Widdowson, M.-A.; Roy, S.L.; Jones, J.L.; Griffin, P.M. Foodborne illness acquired in the united states—major pathogens. *Emerg. Infect. Dis.* **2011**, *17*, 16–22.
5. Majowicz, S.E.; Musto, J.; Scallan, E.; Angulo, F.J.; Kirk, M.; O'Brien, S.J.; Jones, T.F.; Fazil, A.; Hoekstra, R.M. The global burden of nontyphoidal *Salmonella* gastroenteritis. *Clin. Infect. Dis.* **2010**, *50*, 882–889.
6. Jones, T.F.; Ingram, L.A.; Cieslak, P.R.; Vugia, D.J.; Tobin-D'Angelo, M.; Hurd, S.; Medus, C.; Cronquist, A.; Angulo, F.J. Salmonellosis outcomes differ substantially by serotype. *J. Infect. Dis.* **2008**, *198*, 109–114.
7. Uzzau, S.; Brown, D.J.; Wallis, T.; Rubino, S.; Leori, G.; Bernard, S.; Casadesús, J.; Platt, D.J.; Olsen, J.E. Host adapted serotypes of *Salmonella enterica*. *Epidemiol. Infect.* **2000**, *125*, 229–255.
8. Song, J.; Gao, X.; Galán, J.E. Structure and function of the *Salmonella* Typhi chimaeric A2B5 typhoid toxin. *Nature* **2013**, *499*, 350–354.
9. Suez, J.; Porwollik, S.; Dagan, A.; Marzel, A.; Schorr, Y.I.; Desai, P.T.; Agmon, V.; McClelland, M.; Rahav, G.; Gal-Mor, O. Virulence gene profiling and pathogenicity characterization of non-typhoidal *Salmonella* accounted for invasive disease in humans. *PLoS ONE* **2013**, *8*, e58449.
10. Gargi, A.; Reno, M.; Blanke, S.R. Bacterial toxin modulation of the eukaryotic cell cycle: Are all cytolethal distending toxins created equally? *Front. Cell. Infect. Microbiol.* **2012**, *2*, 124.
11. Spanò, S.; Ugalde, J.E.; Galán, J.E. Delivery of a *Salmonella* Typhi exotoxin from a host intracellular compartment. *Cell Host Microbe* **2008**, *3*, 30–38.
12. Haghjoo, E.; Galán, J.E. *Salmonella* Typhi encodes a functional cytolethal distending toxin that is delivered into host cells by a bacterial-internalization pathway. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 4614–4619.
13. Den Bakker, H.C.; Switt, A.I.M.; Govoni, G.; Cummings, C.A.; Ranieri, M.L.; Degoricija, L.; Hoelzer, K.; Rodriguez-Rivera, L.D.; Brown, S.; Bolchacova, E. Genome sequencing reveals diversification of virulence factor content and possible host adaptation in distinct subpopulations of *Salmonella enterica*. *BMC Genom.* **2011**, *12*, 425.
14. Johnson, W.; Lior, H. Response of chinese hamster ovary cells to a cytolethal distending toxin (CDT) of *Escherichia coli* and possible misinterpretation as heat-labile (LT) enterotoxin. *FEMS Microbiol. Lett.* **1987**, *43*, 19–23.
15. Asakura, M.; Samosornsuk, W.; Taguchi, M.; Kobayashi, K.; Misawa, N.; Kusumoto, M.; Nishimura, K.; Matsuhisa, A.; Yamasaki, S. Comparative analysis of cytolethal distending toxin (CDT) genes among *Campylobacter jejuni*, *C. coli* and *C. fetus* strains. *Microb. Pathog.* **2007**, *42*, 174–183.

16. Whitehouse, C.A.; Balbo, P.B.; Pesci, E.C.; Cottle, D.L.; Mirabito, P.M.; Pickett, C.L. *Campylobacter jejuni* cytolethal distending toxin causes a G2-phase cell cycle block. *Infect. Immun.* **1998**, *66*, 1934–1940.
17. Mooney, A.; Clyne, M.; Curran, T.; Doherty, D.; Kilmartin, B.; Bourke, B. *Campylobacter upsaliensis* exerts a cytolethal distending toxin effect on Hela cells and T lymphocytes. *Microbiology* **2001**, *147*, 735–743.
18. Kamei, K.; Asakura, M.; Somroop, S.; Hatanaka, N.; Hinenoya, A.; Nagita, A.; Misawa, N.; Matsuda, M.; Nakagawa, S.; Yamasaki, S. A PCR-RFLP assay for the detection and differentiation of *Campylobacter jejuni*, *C. coli*, *C. fetus*, *C. hyointestinalis*, *C. lari*, *C. helveticus* and *C. upsaliensis*. *J. Med. Microbiol.* **2014**, *63*, 659–666.
19. Cope, L.D.; Lumbley, S.; Latimer, J.L.; Klesney-Tait, J.; Stevens, M.K.; Johnson, L.S.; Purven, M.; Munson, R.S.; Lagergard, T.; Radolf, J.D. A diffusible cytotoxin of *Haemophilus ducreyi*. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 4056–4061.
20. Zhou, M.; Zhang, Q.; Zhao, J.; Jin, M. *Haemophilus parasuis* encodes two functional cytolethal distending toxins: CdtC contains an atypical cholesterol recognition/interaction region. *PLoS ONE* **2012**, *7*, e32580.
21. Belibasakis, G.N.; Mattsson, A.; Wang, Y.; Chen, C.; Johansson, A. Cell cycle arrest of human gingival fibroblasts and periodontal ligament cells by *Actinobacillus actinomycetemcomitans*: Involvement of the cytolethal distending toxin. *Apmis* **2004**, *112*, 674–685.
22. Young, V.B.; Knox, K.A.; Schauer, D.B. Cytolethal distending toxin sequence and activity in the enterohepatic pathogen *Helicobacter hepaticus*. *Infect. Immun.* **2000**, *68*, 184–191.
23. Taylor, N.S.; Ge, Z.; Shen, Z.; Dewhirst, F.E.; Fox, J.G. Cytolethal distending toxin: A potential virulence factor for *Helicobacter cinaedi*. *J. Infect. Dis.* **2003**, *188*, 1892–1897.
24. Young, V.B.; Chien, C.-C.; Knox, K.A.; Taylor, N.S.; Schauer, D.B.; Fox, J.G. Cytolethal distending toxin in avian and human isolates of *Helicobacter pullorum*. *J. Infect. Dis.* **2000**, *182*, 620–623.
25. Liyanage, N.P.; Dassanayake, R.P.; Kuszynski, C.A.; Duhamel, G.E. Contribution of *Helicobacter hepaticus* cytolethal distending toxin subunits to human epithelial cell cycle arrest and apoptotic death *in vitro*. *Helicobacter* **2013**, *18*, 433–443.
26. Cortes-Bratti, X.; Frisan, T.; Thelestam, M. The cytolethal distending toxins induce DNA damage and cell cycle arrest. *Toxicon* **2001**, *39*, 1729–1736.
27. Okuda, J.; Fukumoto, M.; Takeda, Y.; Nishibuchi, M. Examination of diarrheagenicity of cytolethal distending toxin: Suckling mouse response to the products of the CdtABC genes of *Shigella dysenteriae*. *Infect. Immun.* **1997**, *65*, 428–433.
28. Shima, A.; Hinenoya, A.; Asakura, M.; Sugimoto, N.; Tsukamoto, T.; Ito, H.; Nagita, A.; Faruque, S.M.; Yamasaki, S. Molecular characterizations of cytolethal distending toxin produced by *Providencia alcalifaciens* strains isolated from patients with diarrhea. *Infect. Immun.* **2012**, *80*, 1323–1332.
29. Parkhill, J.; Dougan, G.; James, K.; Thomson, N.; Pickard, D.; Wain, J.; Churcher, C.; Mungall, K.; Bentley, S.; Holden, M. Complete genome sequence of a multiple drug resistant *Salmonella enterica* serovar Typhi CT18. *Nature* **2001**, *413*, 848–852.
30. Locht, C.; Coutte, L.; Mielcarek, N. The ins and outs of pertussis toxin. *FEBS J.* **2011**, *278*, 4668–4682.
31. Paton, A.W.; Paton, J.C. *Escherichia coli* subtilase cytotoxin. *Toxins* **2010**, *2*, 215–228.

32. Mezal, E.H.; Bae, D.; Khan, A.A. Detection and functionality of the CdtB, PltA, and PltB from *Salmonella enterica* serovar Javiana. *Pathog. Dis.* **2014**, *72*, 95–103.
33. Williams, K.; Gokulan, K.; Shelman, D.; Akiyama, T.; Khan, A.; Khare, S. Cytotoxic mechanism of cytolethal distending toxin in nontyphoidal *Salmonella* serovar (*Salmonella* Javiana) during macrophage infection. *DNA Cell Biol.* **2015**, *34*, 113–124.
34. Rodriguez-Rivera, L.D.; Bowen, B.M.; den Bakker, H.C.; Duhamel, G.E.; Wiedmann, M. Characterization of the cytolethal distending toxin (typhoid toxin) in non-typhoidal salmonella serovars. *Gut Pathog.* **2015**, *7*, doi:10.1186/s13099-015-0065-1.
35. Desai, P.T.; Porwollik, S.; Long, F.; Cheng, P.; Wollam, A.; Clifton, S.W.; Weinstock, G.M.; McClelland, M. Evolutionary genomics of *Salmonella enterica* subspecies. *mBio* **2013**, *4*, e00579-12.
36. Allard, M.W.; Luo, Y.; Strain, E.; Li, C.; Keys, C.E.; Son, I.; Stones, R.; Musser, S.M.; Brown, E.W. High resolution clustering of *Salmonella enterica* serovar Montevideo strains using a next-generation sequencing approach. *BMC Genom.* **2012**, *13*, doi:10.1186/1471-2164-13-32.
37. Tan, K.S.; Ong, G.; Song, K.P. Introns in the cytolethal distending toxin gene of *Actinobacillus actinomycetemcomitans*. *J. Bacteriol.* **2005**, *187*, 567–575.
38. Nakajima, T.; Tazumi, A.; Hirayama, J.; Hayashi, K.; Tasaki, E.; Asakura, M.; Yamasaki, S.; Moore, J.; Millar, B.; Matsubara, K. Expression and analysis of a cytolethal distending toxin (CDT) gene operon in *Campylobacter lari*. *Br. J. Biomed. Sci.* **2012**, *69*, 26.
39. Haghjoo, E.; Galán, J.E. Identification of a transcriptional regulator that controls intracellular gene expression in *Salmonella* Typhi. *Mol. Microbiol.* **2007**, *64*, 1549–1561.
40. Guidi, R.; Levi, L.; Rouf, S.F.; Puiac, S.; Rhen, M.; Frisan, T. *Salmonella enterica* delivers its genotoxin through outer membrane vesicles secreted from infected cells. *Cell. Microbiol.* **2013**, *15*, 2034–2050.
41. Charles, R.C.; Harris, J.B.; Chase, M.R.; Lebrun, L.M.; Sheikh, A.; LaRocque, R.C.; Logvinenko, T.; Rollins, S.M.; Tarique, A.; Hohmann, E.L. Comparative proteomic analysis of the PhoP regulon in *Salmonella enterica* serovar Typhi *versus* Typhimurium. *PLoS ONE* **2009**, *4*, e6994.
42. Hodak, H.; Galan, J.E. A *Salmonella* Typhi homologue of bacteriophage muramidases controls typhoid toxin secretion. *EMBO Rep.* **2013**, *14*, 95–102.
43. Saitoh, M.; Tanaka, K.; Nishimori, K.; Makino, S.-i.; Kanno, T.; Ishihara, R.; Hatama, S.; Kitano, R.; Kishima, M.; Sameshima, T. The artAB genes encode a putative ADP-ribosyltransferase toxin homologue associated with *Salmonella enterica* serovar Typhimurium DT104. *Microbiology* **2005**, *151*, 3089–3096.
44. UniProtKB–Q8Z6A3 (Q8Z6A3_SALTI). Available online: http://www.uniprot.org/uniprot/Q8Z6A3 (accessed on 3 March 2016).
45. Wang, H.; Paton, J.C.; Herdman, B.P.; Rogers, T.J.; Beddoe, T.; Paton, A.W. The B subunit of an AB5 toxin produced by *Salmonella enterica* serovar Typhi up-regulates chemokines, cytokines, and adhesion molecules in human macrophage, colonic epithelial, and brain microvascular endothelial cell lines. *Infect. Immun.* **2013**, *81*, 673–683.
46. Hazes, B.; Boodhoo, A.; Cockle, S.A.; Read, R.J. Crystal structure of the pertussis toxin–ATP complex: A molecular sensor. *J. Mol. Biol.* **1996**, *258*, 661–671.
47. Nešić, D.; Hsu, Y.; Stebbins, C.E. Assembly and function of a bacterial genotoxin. *Nature* **2004**, *429*, 429–433.

48. UniProtKB–Q8Z6A4 (Q8Z6A4_SALTI). Available online: http://www.uniprot.org/uniprot/Q8Z6A4 (accessed on 26 January 2016).
49. Carbonetti, N.H. Pertussis toxin and adenylate cyclase toxin: Key virulence factors of *Bordetella pertussis* and cell biology tools. *Future Microbiol.* **2010**, *5*, 455–469.
50. Aktories, K. Bacterial protein toxins that modify host regulatory GTPases. *Nat. Rev. Microbiol.* **2011**, *9*, 487–498.
51. Jinadasa, R.N.; Bloom, S.E.; Weiss, R.S.; Duhamel, G.E. Cytolethal distending toxin: A conserved bacterial genotoxin that blocks cell cycle progression, leading to apoptosis of a broad range of mammalian cell lineages. *Microbiology* **2011**, *157*, 1851–1875.
52. Shenker, B.J.; Dlakić, M.; Walker, L.P.; Besack, D.; Jaffe, E.; LaBelle, E.; Boesze-Battaglia, K. A novel mode of action for a microbial-derived immunotoxin: The cytolethal distending toxin subunit B exhibits phosphatidylinositol 3, 4, 5-triphosphate phosphatase activity. *J. Immunol.* **2007**, *178*, 5099–5108.
53. DiRienzo, J.M. Uptake and processing of the cytolethal distending toxin by mammalian cells. *Toxins* **2014**, *6*, 3098–3116.
54. Akifusa, S.; Heywood, W.; Nair, S.P.; Stenbeck, G.; Henderson, B. Mechanism of internalization of the cytolethal distending toxin of *Actinobacillus actinomycetemcomitans*. *Microbiology* **2005**, *151*, 1395–1402.
55. Eshraghi, A.; Maldonado-Arocho, F.J.; Gargi, A.; Cardwell, M.M.; Prouty, M.G.; Blanke, S.R.; Bradley, K.A. Cytolethal distending toxin family members are differentially affected by alterations in host glycans and membrane cholesterol. *J. Biol. Chem.* **2010**, *285*, 18199–18207.
56. Gargi, A.; Tamilselvam, B.; Powers, B.; Prouty, M.G.; Lincecum, T.; Eshraghi, A.; Maldonado-Arocho, F.J.; Wilson, B.A.; Bradley, K.A.; Blanke, S.R. Cellular interactions of the cytolethal distending toxins from *Escherichia coli* and *Haemophilus ducreyi*. *J. Biol. Chem.* **2013**, *288*, 7492–7505.
57. McSweeney, L.A.; Dreyfus, L.A. Carbohydrate-binding specificity of the *Escherichia coli* cytolethal distending toxin CdtA and CdtC subunits. *Infect. Immun.* **2005**, *73*, 2051–2060.
58. Mise, K.; Akifusa, S.; Watarai, S.; Ansai, T.; Nishihara, T.; Takehara, T. Involvement of ganglioside GM3 in G2/M cell cycle arrest of human monocytic cells induced by *Actinobacillus actinomycetemcomitans* cytolethal distending toxin. *Infect. Immun.* **2005**, *73*, 4846–4852.
59. Guerra, L.; Cortes-Bratti, X.; Guidi, R.; Frisan, T. The biology of the cytolethal distending toxins. *Toxins* **2011**, *3*, 172–190.
60. Chong, D.C.; Paton, J.C.; Thorpe, C.M.; Paton, A.W. Clathrin-dependent trafficking of subtilase cytotoxin, a novel AB5 toxin that targets the endoplasmic reticulum chaperone BiP. *Cell. Microbiol.* **2008**, *10*, 795–806.
61. Guerra, L.; Teter, K.; Lilley, B.N.; Stenerlöw, B.; Holmes, R.K.; Ploegh, H.L.; Sandvig, K.; Thelestam, M.; Frisan, T. Cellular internalization of cytolethal distending toxin: A new end to a known pathway. *Cell. Microbiol.* **2005**, *7*, 921–934.
62. Eshraghi, A.; Dixon, S.D.; Tamilselvam, B.; Kim, E.J.-K.; Gargi, A.; Kulik, J.C.; Damoiseaux, R.; Blanke, S.R.; Bradley, K.A. Cytolethal distending toxins require components of the ER-associated degradation pathway for host cell entry. *PLOS Pathog*. **2014**, doi:10.1371/journal.ppat.1004295.
63. Elliott, R.M. Orthobunyaviruses: Recent genetic and structural insights. *Nat. Rev. Microbiol.* **2014**, *12*, 673–685.

64. Fedor, Y.; Vignard, J.; Nicolau-Travers, M.L.; Boutet-Robinet, E.; Watrin, C.; Salles, B.; Mirey, G. From single-strand breaks to double-strand breaks during s-phase: A new mode of action of the *Escherichia coli* cytolethal distending toxin. *Cell. Microbiol.* **2013**, *15*, 1–15.
65. Bezine, E.; Vignard, J.; Mirey, G. The cytolethal distending toxin effects on mammalian cells: A DNA damage perspective. *Cells* **2014**, *3*, 592–615.
66. Xu, B.; Kim, S.-T.; Lim, D.-S.; Kastan, M.B. Two molecularly distinct G2/M checkpoints are induced by ionizing irradiation. *Mol. Cell. Biol.* **2002**, *22*, 1049–1059.
67. Shenker, B.J.; Demuth, D.R.; Zekavat, A. Exposure of lymphocytes to high doses of *Actinobacillus actinomycetemcomitans* cytolethal distending toxin induces rapid onset of apoptosis-mediated DNA fragmentation. *Infect. and Immun.* **2006**, *74*, 2080–2092.
68. Mangerich, A.; Knutson, C.G.; Parry, N.M.; Muthupalani, S.; Ye, W.; Prestwich, E.; Cui, L.; McFaline, J.L.; Mobley, M.; Ge, Z. Infection-induced colitis in mice causes dynamic and tissue-specific changes in stress response and DNA damage leading to colon cancer. *Proc. Natl. Acad. Sci.* **2012**, *109*, E1820–E1829.
69. Hassane, D.C.; Lee, R.B.; Pickett, C.L. *Campylobacter jejuni* cytolethal distending toxin promotes DNA repair responses in normal human cells. *Infect. Immun.* **2003**, *71*, 541–545.
70. Alaoui-El-Azher, M.; Mans, J.J.; Baker, H.V.; Chen, C.; Progulske-Fox, A.; Lamont, R.J.; Handfield, M. Role of the ATM-checkpoint kinase 2 pathway in CDT-mediated apoptosis of gingival epithelial cells. *PLoS ONE* **2010**, *5*, e11714.
71. Li, L.; Sharipo, A.; Chaves-Olarte, E.; Masucci, M.G.; Levitsky, V.; Thelestam, M.; Frisan, T. The *Haemophilus ducreyi* cytolethal distending toxin activates sensors of DNA damage and repair complexes in proliferating and non-proliferating cells. *Cell. Microbiol.* **2002**, *4*, 87–99.
72. Kato, S.; Nakashima, K.; Nagasawa, T.; Abiko, Y.; Furuichi, Y. Involvement of toll-like receptor 2 in apoptosis of *Aggregatibacter actinomycetemcomitans*-infected Thp-1 cells. *J. Microbiol. Immunol. Infect.* **2013**, *46*, 164–170.
73. Rabin, S.D.; Flitton, J.G.; Demuth, D.R. *Aggregatibacter actinomycetemcomitans* cytolethal distending toxin induces apoptosis in nonproliferating macrophages by a phosphatase-independent mechanism. *Infect. immun.* **2009**, *77*, 3161–3169.
74. Cortes-Bratti, X.; Karlsson, C.; Lagergård, T.; Thelestam, M.; Frisan, T. The *Haemophilus ducreyi* cytolethal distending toxin induces cell cycle arrest and apoptosis via the DNA damage checkpoint pathways. *J. Biol. Chem.* **2001**, *276*, 5296–5302.
75. Hickey, T.E.; Majam, G.; Guerry, P. Intracellular survival of *Campylobacter jejuni* in human monocytic cells and induction of apoptotic death by cytholethal distending toxin. *Infect. Immun.* **2005**, *73*, 5194.
76. Bielaszewska, M.; Sinha, B.; Kuczius, T.; Karch, H. Cytolethal distending toxin from shiga toxin-producing *Escherichia coli* O157 causes irreversible G2/M arrest, inhibition of proliferation, and death of human endothelial cells. *Infect Immun.* **2005**, *73*, 552–562.
77. Liyanage, N.P.; Manthey, K.C.; Dassanayake, R.P.; Kuszynski, C.A.; Oakley, G.G.; Duhamel, G.E. *Helicobacter hepaticus* cytolethal distending toxin causes cell death in intestinal epithelial cells via mitochondrial apoptotic pathway. *Helicobacter* **2010**, *15*, 98–107.
78. Asakura, H.; Momose, Y.; Ryu, C.-H.; Kasuga, F.; Yamamoto, S.; Kumagai, S.; Igimi, S. *Providencia alcalifaciens* causes barrier dysfunction and apoptosis in tissue cell culture: Potent role of lipopolysaccharides on diarrheagenicity. *Food Add. Contam. Part A* **2013**, *30*, 1459–1466.
79. Guidi, R.; Guerra, L.; Levi, L.; Stenerlöw, B.; Fox, J.G.; Josenhans, C.; Masucci, M.G.; Frisan, T. Chronic exposure to the cytolethal distending toxins of Gram-negative bacteria

promotes genomic instability and altered DNA damage response. *Cell.Microbiol.* **2013**, *15*, 98–113.
80. Karin, M.; Lawrence, T.; Nizet, V. Innate immunity gone awry: Linking microbial infections to chronic inflammation and cancer. *Cell* **2006**, *124*, 823–835.
81. Shen, Z.; Feng, Y.; Rogers, A.; Rickman, B.; Whary, M.; Xu, S.; Clapp, K.; Boutin, S.; Fox, J. Cytolethal distending toxin promotes *Helicobacter cinaedi*-associated typhlocolitis in interleukin-10-deficient mice. *Infect. Immun.* **2009**, *77*, 2508–2516.
82. Ge, Z.; Rogers, A.B.; Feng, Y.; Lee, A.; Xu, S.; Taylor, N.S.; Fox, J.G. Bacterial cytolethal distending toxin promotes the development of dysplasia in a model of microbially induced hepatocarcinogenesis. *Cell. microbiol.* **2007**, *9*, 2070–2080.
83. Ge, Z.; Feng, Y.; Whary, M.T.; Nambiar, P.R.; Xu, S.; Ng, V.; Taylor, N.S.; Fox, J.G. Cytolethal distending toxin is essential for *Helicobacter hepaticus* colonization in outbred swiss webster mice. *Infect. Immun.* **2005**, *73*, 3559–3567.
84. Guerra, L.; Nemec, K.N.; Massey, S.; Tatulian, S.A.; Thelestam, M.; Frisan, T.; Teter, K. A novel mode of translocation for cytolethal distending toxin. *Biochim. Biophys Acta (BBA)-Mol. Cell Res.* **2009**, *1793*, 489–495.
85. Fahrer, J.; Huelsenbeck, J.; Jaurich, H.; Dörsam, B.; Frisan, T.; Eich, M.; Roos, W.P.; Kaina, B.; Fritz, G. Cytolethal distending toxin (cdt) is a radiomimetic agent and induces persistent levels of DNA double-strand breaks in human fibroblasts. *DNA Repair* **2014**, *18*, 31–43.
86. Buc, E.; Dubois, D.; Sauvanet, P.; Raisch, J.; Delmas, J.; Darfeuille-Michaud, A.; Pezet, D.; Bonnet, R. High prevalence of mucosa-associated *E. coli* producing cyclomodulin and genotoxin in colon cancer. *PLoS ONE* **2013**, *8*, e56964.
87. Nalbant, A.; Zadeh, H. *Actinobacillus actinomycetemcomitans* induces apoptosis of T lymphocytes by the Fas and Fas ligand pathway. *Oral microbiol. Immunol.* **2002**, *17*, 277–284.
88. Ohara, M.; Hayashi, T.; Kusunoki, Y.; Miyauchi, M.; Takata, T.; Sugai, M. Caspase-2 and caspase-7 are involved in cytolethal distending toxin-induced apoptosis in Jurkat and MOLT-4 T-cell lines. *Infect. Immun.* **2004**, *72*, 871–879.
89. Shenker, B.J.; Hoffmaster, R.H.; Zekavat, A.; Yamaguchi, N.; Lally, E.T.; Demuth, D.R. Induction of apoptosis in human t cells by *Actinobacillus actinomycetemcomitans* cytolethal distending toxin is a consequence of G2 arrest of the cell cycle. *J. Immunol.* **2001**, *167*, 435–441.
90. Wising, C.; Azem, J.; Zetterberg, M.; Svensson, L.A.; Ahlman, K.; Lagergård, T. Induction of apoptosis/necrosis in various human cell lineages by *Haemophilus ducreyi* cytolethal distending toxin. *Toxicon* **2005**, *45*, 767–776.
91. Shacter, E.; Weitzman, S.A. Chronic inflammation and cancer. *Oncology* **2002**, *16*, 217–232.
92. Arthur, J.C.; Perez-Chanona, E.; Mühlbauer, M.; Tomkovich, S.; Uronis, J.M.; Fan, T.-J.; Campbell, B.J.; Abujamel, T.; Dogan, B.; Rogers, A.B. Intestinal inflammation targets cancer-inducing activity of the microbiota. *Science* **2012**, *338*, 120–123.
93. Mantovani, A.; Allavena, P.; Sica, A.; Balkwill, F. Cancer-related inflammation. *Nature* **2008**, *454*, 436–444.
94. Scanu, T.; Spaapen, R.M.; Bakker, J.M.; Pratap, C.B.; Wu, L.; Hofland, I.; Broeks, A.; Shukla, V.K.; Kumar, M.; Janssen, H. *Salmonella* manipulation of host signaling pathways provokes cellular transformation associated with gallbladder carcinoma. *Cell Host Microbe* **2015**, *17*, 763–774.

95. Gonzalez-Escobedo, G.; Marshall, J.M.; Gunn, J.S. Chronic and acute infection of the gall bladder by *Salmonella* Typhi: Understanding the carrier state. *Nat. Rev. Microbiol.* **2011**, *9*, 9–14.
96. Nagaraja, V.; Eslick, G. Systematic review with meta-analysis: The relationship between chronic *Salmonella* Typhi carrier status and gall-bladder cancer. *Aliment. Pharmacol. Ther.* **2014**, *39*, 745–750.
97. Song, J.; Willinger, T.; Rongvaux, A.; Eynon, E.E.; Stevens, S.; Manz, M.G.; Flavell, R.A.; Galán, J.E. A mouse model for the human pathogen *Salmonella* Typhi. *Cell Host Microbe* **2010**, *8*, 369–376.
98. Sabbagh, S.C.; Forest, C.G.; Lepage, C.; Leclerc, J.-M.; Daigle, F. So similar, yet so different: Uncovering distinctive features in the genomes of *Salmonella enterica* serovars Typhimurium and Typhi. *FEMS Microbiol. Lett.* **2010**, *305*, 1–13.
99. De Jong, H.K.; Parry, C.M.; van der Poll, T.; Wiersinga, W.J. Host–pathogen interaction in invasive salmonellosis. *PloS Pathog.* **2012**, doi:10.1371/journal.ppat.1002933.
100. Jones, B.D.; Falkow, S. Salmonellosis: Host immune responses and bacterial virulence determinants. *Annu. Rev. Immunol.* **1996**, *14*, 533–561.
101. Levine, M.M.; Black, R.E.; Lanata, C. Precise estimation of the numbers of chronic carriers of *Salmonella* Typhi in Santiago, Chile, an endemic area. *J. Infect. Dis.* **1982**, *146*, 724–726.
102. Merselis, J.G.; Kaye, D.; Connolly, C.S.; Hook, E.W. Quantitative bacteriology of the typhoid carrier state. *Am. J. Trop. Med. Hyg.* **1964**, *13*, 425–429.
103. Gunn, J.S.; Marshall, J.M.; Baker, S.; Dongol, S.; Charles, R.C.; Ryan, E.T. *Salmonella* chronic carriage: Epidemiology, diagnosis, and gallbladder persistence. *Trends Microbiol.* **2014**, *22*, 648–655.
104. Kariuki, S.; Revathi, G.; Kariuki, N.; Kiiru, J.; Mwituria, J.; Muyodi, J.; Githinji, J.W.; Kagendo, D.; Munyalo, A.; Hart, C.A. Invasive multidrug-resistant non-typhoidal *Salmonella* infections in Africa: Zoonotic or anthroponotic transmission? *J. Med. Microbiol.* **2006**, *55*, 585–591.
105. Lax, A.J. Bacterial toxins and cancer—A case to answer? *Nat. Rev. Microbiol.* **2005**, *3*, 343–349.
106. Kåhrström, C.T. Bacterial pathogenesis: *E. coli* claims the driving seat for cancer. *Nat. Rev. Microbiol.* **2012**, *10*, 670–671.
107. Gould, L.H.; Bopp, C.; Strockbine, N.; Atkinson, R.; Baselski, V.; Body, B.; Carey, R.; Crandall, C.; Hurd, S.; Kaplan, R. Recommendations for diagnosis of shiga toxin–Producing *Escherichia coli* infections by clinical laboratories. *MMWR Recomm. Rep.* **2009**, *58*, 1-14.
108. Wong, C.S.; Jelacic, S.; Habeeb, R.L.; Watkins, S.L.; Tarr, P.I. The risk of the hemolytic–uremic syndrome after antibiotic treatment of *Escherichia coli* O157:H7 infections. *N. Engl. J Med.* **2000**, *342*, 1930–1936.
109. Lowy, I.; Molrine, D.C.; Leav, B.A.; Blair, B.M.; Baxter, R.; Gerding, D.N.; Nichol, G.; Thomas, W.D., Jr.; Leney, M.; Sloan, S. Treatment with monoclonal antibodies against *Clostridium difficile* toxins. *N. Engl. J Med.* **2010**, *362*, 197.
110. Sobel, J. Botulism. *Clin. Infect. Dis.* **2005**, *41*, 1167–1173.
111. Goldwater, P.N.; Bettelheim, K.A. Treatment of enterohemorrhagic *Escherichia coli* (EHEC) infection and hemolytic uremic syndrome (HUS). *BMC Med.* **2012**, *10*, 1.

CHAPTER 3

THE CYTOLETHAL DISTENDING TOXIN PRODUCED BY NONTYPHOIDAL SALMONELLA SEROTYPES JAVIANA, MONTEVIDEO, ORANIENBURG, AND MISSISSIPPI, INDUCES DNA DAMAGE IN A MANNER SIMILAR TO SEROTYPE TYPHI*

*****R.A. Miller, and M. Wiedmann

Published in mBio 2016, 7(6):e02109-16. doi:10.1128/mBio.02109-16

ABSTRACT

Select nontyphoidal *Salmonella* (NTS) serotypes were recently found to encode the *Salmonella* cytolethal distending toxin (S-CDT), an important virulence factor for serotype Typhi, the causative agent of typhoid fever. Using a PCR-based assay we determined that among 21 NTS serotypes causing the majority of foodborne salmonellosis cases in the US, genes encoding S-CDT are conserved in isolates representing serotypes Javiana, Montevideo, and Oranienburg, while for serotype Mississippi isolates the presence of S-CDT-encoding genes is clade-associated. HeLa cells infected with representative strains of these S-CDT-positive serotypes had a significantly higher proportion of cells arrested in the G2/M phase compared to HeLa cells infected with representative strains of S-CDT-negative serotypes Typhimurium, Newport, and Enteritidis. The G2/M cell cycle arrest was dependent on CdtB, the active subunit of S-CDT, as infection with isogenic Δ*cdtB* mutants abolished their ability to induce a G2/M cell cycle arrest. Infection with S-CDT-encoding serotypes was significantly associated with activation of the host cell's DNA damage response (DDR), a signaling cascade that is important for detecting and repairing damaged DNA. HeLa cell populations infected with S-CDT-positive serotypes had a significantly higher proportion of cells with DDR proteins 53BP1 and γH2AX foci, compared to cells infected with either S-CDT-negative serotypes or isogenic Δ*cdtB* strains. Intoxication with S-CDT occurred via autocrine and paracrine pathways, as uninfected HeLa cells among populations of infected cells also had an activated DDR. Overall, we show that S-CDT plays a significant role in the cellular outcome of infection with NTS serotypes.

IMPORTANCE

The recent discovery that multiple serotypes encode S-CDT, which was previously established as an important virulence factor for serotype Typhi, suggested that this toxin may also contribute to the outcome of infection with nontyphoidal serotypes. In this study, we demonstrate that at a cellular level, S-CDT significantly alters the outcome of infection by inducing DNA damage which is associated with a cell cycle arrest and activation of the host cell's DDR. Importantly, these results contribute valuable information for assessing the public

health implications of S-CDT in infections with NTS serotypes. Our data suggest that infection with *Salmonella* strains that encode S-CDT has the potential to result in DNA damage, which may contribute to long-term sequelae.

INTRODUCTION

Cytolethal distending toxins (CDT) are important virulence factors produced by Gram-negative bacteria, including those causing predominantly extracellular infections (*Aggregatibacter actinomycetemcomitans*, *Haemophilus* spp., *Providencia alcalifaciens*) and those able to cause intracellular infections (*Campylobacter* spp., *Escherichia coli*, *Helicobacter* spp., *Salmonella enterica*, *Shigella* spp., and *Yersinia* spp.) (1, 2). *In vitro*, CDTs act as genotoxins, inducing single strand or double strand breaks, resulting in activation of the eukaryotic cell DDR (3-5). DDR protein 53 binding protein 1 (53BP1), and phosphorylation of histone 2AX (producing γH2AX), have been shown previously to localize at DNA double strand breaks, recruiting DNA repair machinery to the DNA damage (6, 7). *In vivo*, CDT-induced damage has been associated with enhanced colonization of the host by CDT-producing pathogens, tumorigenesis and neoplastic lesions, as well as contributing to the establishment of chronic infections (8-10).

The CDTs encoded by most Gram-negative pathogens exist as an AB_2 toxin composed of 2 binding subunits, CdtA and CdtC, and an active subunit CdtB (3). *In vitro* analyses have demonstrated nuclease activity of the CdtB subunit using plasmid relaxation assays (11). However, the CDT encoded by select *S. enterica* serotypes (referred to as S-CDT for *Salmonella* cytolethal distending toxin) represents a unique form of CDT with an A_2B_5 configuration with 2 active subunits (CdtB and PltA), and 5 binding subunits (PltB) arranged as a pentameric ring (2, 12). The PltA subunit shares structural and functional homology with the S1 subunit of the pertussis toxin, which acts as an ADP-ribosyl transferase (2, 13). The PltB subunit has been suggested to play a role in binding of S-CDT to host cell receptors, as it shares homology to the binding subunits of both the pertussis toxin (subunits S2 and S3) and the subtilase cytotoxin (SubB) produced by *E. coli* (12-14).

S-CDT was originally characterized as a unique virulence factor of *S. enterica* subspecies *enterica* serotype Typhi (2, 15, 16). *In vitro* studies demonstrated that, similar to CDTs produced by other Gram-negative pathogens, the S-CDT produced by *S.* Typhi induced DNA damage leading to a G2/M arrest among infected cell culture populations (2, 12, 16). Recent studies suggest that the S-CDT produced by *S.* Typhi is capable of recapitulating symptoms of typhoid fever, modulating the immune system, and enabling *S.* Typhi to persist *in vivo* (8, 12).

Each year, NTS infections account for approximately 1.03 million cases of foodborne illness in the US, and an estimated 80.3 million cases internationally (17, 18). There are marked differences in the disease severity resulting from infections with different *Salmonella* serotypes (19), yet genetic analyses have failed to definitively account for differences in virulence among nontyphoidal *Salmonella* (NTS) serotypes (20, 21). Recent studies have identified genes encoding S-CDT in > 40 NTS serotypes (20, 22). However little is known regarding the distribution and conservation of S-CDT-encoding genes among NTS serotypes, and the contributions of S-CDT in the course of infection with NTS serotypes (23-25).

In this study, we examined differences in the cellular outcome of infection between S-CDT-positive and negative NTS serotypes. Using a PCR-based screen, we characterized the distribution and sequence conservation of S-CDT-encoding genes among 21 of the NTS serotypes most frequently isolated from clinical cases of salmonellosis in the US. Next, we examined the cellular response to infection with the 4 S-CDT-positive serotypes identified through our PCR-screen, and compared this to infection with 3 S-CDT-negative serotypes in order to determine what role S-CDT may play in a cell model of infection. Overall, we show that genes encoding S-CDT are highly conserved among isolates representing NTS serotypes Javiana, Montevideo, Oranienburg, as well as 1 clade of serotype Mississippi. Importantly, infection with NTS serotypes encoding S-CDT significantly alters the outcome of infection in a HeLa cell model. These data suggest that S-CDT is an important virulence factor in NTS, which may contribute to differences in disease severity observed among different NTS serotypes. With the recognized potential of CDTs to serve as genotoxins, our data also suggest that infection with

some *Salmonella* strains (i.e., those that carry S-CDT) has the potential to cause DNA damage in host cells, which may predispose case patients to long-term sequelae that remain to be defined.

MATERIALS AND METHODS

Bacterial strains, human cell lines, and culturing conditions. A total of 864 wild-type *Salmonella* isolates obtained from foods (e.g., raw oysters, shrimp, jalapeño peppers, spices, and others), environmental samples (e.g., ground water, soil, drag swabs from produce fields, dairy and avian farm environments), animals (e.g., bovines, avians, reptiles), and humans with clinical symptoms in the US were selected from an existing strain collection for inclusion in this study (see Table 3.1 and Table 3.2 for complete list).

TABLE 3.1 *Salmonella* strains used for *in vitro* analyses

Strain	Serotype	Genotype	Source	Date Isolated[a]
FSL S5-0415	Enteritidis	Wild-type	Human	April 2004
FSL S5-0536	Typhimurium	Wild-type	Human	November 2004
FSL S5-0639	Newport	Wild-type	Human	December 2004
FSL S5-0395	Javiana	Wild-type	Human	March 2004
FSL R8-2779	Mississippi	Wild-type	Human	November 2008
FSL R8-4841	Montevideo	Wild-type	Human	April 2010
FSL S5-0642	Oranienburg	Wild-type	Human	December 2004
FSL B2-0360	Javiana	FSL S5-0395 Δ*cdtB*	Lab Strain	NA[b]
FSL B2-0364	Montevideo	FSL R8-4841 Δ*cdtB*	Lab Strain	NA[b]

[a]NA= not applicable
[b]these mutants and their construction were previously described (25)

Isolates were selected to belong to 21 NTS serotypes most frequently isolated from human clinical cases of salmonellosis in the US in 2011, as reported by the 2011 CDC National Salmonella Surveillance report. Approximately 50 isolates were selected for each serotype except for 7 serotypes (see Table 2.2) where only a smaller number of isolates could be obtained from the Cornell Food Safety Laboratory's *Salmonella* collection. In addition, the isogenic Δ*cdtB* mutants FSL B2-0360 and FSL B2-0364 and the corresponding parent strains FSL S5-0395 (serotype Javiana) and FSL R8-4181 (serotype Montevideo) were used (Table 2.1); these

strains including the Δ*cdtB* mutants had previously been described (25). As previously detailed, Δ*cdtB* mutants were constructed by deleting the entire *cdtB* ORF using the λ-Red recombinase method (26). All strains were maintained in 15% v/v glycerol stored at -80°C. *Salmonella* were grown on brain heart infusion (BHI; Becton Dickinson, Sparks, MD) agar plates, which were incubated at 37°C. For infections, *Salmonella* strains were cultured in Lysogeny Broth (LB; pH 8, 0.3 M NaCl) under static conditions at 37°C to increase invasion efficiency (27-29).

TABLE 2.3 All Salmonella isolates screened for *pltA*, *pltB*, and *cdtB*

Isolate ID	Serotype	Source	16s (Y/N)	*pltA* (Y/N)	*cdtB* (Y/N)	*pltB* (Y/N)
FSL A4-0021	Agona	animal	Y	N	N	N
FSL A4-0656	Agona	human	Y	N	N	N
FSL A4-0792	Agona	animal	Y	N	N	N
FSL M5-0005	Agona	animal	Y	N	N	N
FSL M8-0482	Agona	environmental swab	Y	N	N	N
FSL M8-0483	Agona	environmental swab	Y	N	N	N
FSL M8-0484	Agona	environmental swab	Y	N	N	N
FSL M8-0485	Agona	nutmeg, ground	Y	N	N	N
FSL M8-0486	Agona	hard cheese	Y	N	N	N
FSL M8-0487	Agona	raw peeled shrimp	Y	N	N	N
FSL M8-0488	Agona	basil, fresh	Y	N	N	N
FSL M8-0490	Agona	Sewage Effluent	Y	N	N	N
FSL R6-0774	Agona	environment, farm	Y	N	N	N
FSL R8-0774	Agona	human, clinical	Y	N	N	N
FSL R8-0779	Agona	human, clinical	Y	N	N	N
FSL R8-1193	Agona	human, clinical	Y	N	N	N
FSL R8-1194	Agona	human, clinical	Y	N	N	N
FSL R8-1195	Agona	human, clinical	Y	N	N	N
FSL R8-1196	Agona	human, clinical	Y	N	N	N
FSL R8-1197	Agona	human, clinical	Y	N	N	N
FSL R8-2497	Agona	human, clinical	Y	N	N	N
FSL R8-2519	Agona	human, clinical	Y	N	N	N
FSL R8-2527	Agona	human, clinical	Y	N	N	N
FSL R8-2566	Agona	human, clinical	Y	N	N	N
FSL R8-2961	Agona	human, clinical	Y	N	N	N
FSL R8-3244	agona	animal, clinical	Y	N	N	N
FSL R8-3344	agona	animal, clinical	Y	N	N	N
FSL R8-3712	Agona	human, clinical	Y	N	N	N
FSL R8-3810	Agona	animal, clinical	Y	N	N	N
FSL R8-3811	Agona	animal, clinical	Y	N	N	N
FSL R8-3819	Agona	animal, clinical	Y	N	N	N
FSL R8-3820	Agona	animal, clinical	Y	N	N	N
FSL R8-3821	Agona	animal, clinical	Y	N	N	N
FSL R8-3822	Agona	animal, clinical	Y	N	N	N
FSL R8-3823	Agona	animal, clinical	Y	N	N	N
FSL R8-3825	Agona	animal, clinical	Y	N	N	N
FSL R8-4666	Agona	human, clinical	Y	N	N	N
FSL R8-5059	Agona	human, clinical	Y	N	N	N
FSL R8-5377	Agona	human, clinical	Y	N	N	N
FSL R8-7953	Agona	human, clinical	Y	N	N	N
FSL R8-8141	Agona	human, clinical	Y	N	N	N
FSL R8-9536	Agona	human, clinical	Y	N	N	N
FSL R9-0830	Agona	human, clinical	Y	N	N	N
FSL S10-1750	Agona	environment, farm	Y	N	N	N
FSL S10-1759	Agona	environment, farm	Y	N	N	N
FSL S10-1760	Agona	environment, farm	Y	N	N	N
FSL S10-1761	Agona	environment, farm	Y	N	N	N
FSL S5-0437	Agona	animal	Y	N	N	N
FSL S5-0547	Agona	animal	Y	N	N	N
FSL S9-0322	Agona	food	Y	N	N	N
FSL A4-0009	Anatum	animal	Y	N	N	N
FSL A4-0525	Anatum	animal	Y	N	N	N
FSL A4-0550	Anatum	animal	Y	N	N	N
FSL A4-0576	Anatum	human	Y	N	N	N
FSL A4-0635	Anatum	human	Y	N	N	N
FSL A4-0660	Anatum	human	Y	N	N	N
FSL R6-0513	Anatum	human, clinical	Y	N	N	N
FSL R6-0788	Anatum	environment, farm	Y	N	N	N
FSL R6-0927	Anatum	environment, farm	Y	N	N	N
FSL R6-0928	Anatum	environment, farm	Y	N	N	N

FSL R8-0187	Anatum	environment, farm	Y	N	N	N
FSL R8-0188	Anatum	environment, farm	Y	N	N	N
FSL R8-0349	Anatum	environment, farm	Y	N	N	N
FSL R8-0350	Anatum	environment, farm	Y	N	N	N
FSL R8-0351	Anatum	environment, farm	Y	N	N	N
FSL R8-0353	Anatum	environment, farm	Y	N	N	N
FSL R8-0354	Anatum	environment, farm	Y	N	N	N
FSL R8-0905	Anatum	environment, farm	Y	N	N	N
FSL R8-0906	Anatum	environment, farm	Y	N	N	N
FSL R8-0907	Anatum	environment, farm	Y	N	N	N
FSL R8-0908	Anatum	environment, farm	Y	N	N	N
FSL R8-1260	anatum	animal, clinical	Y	N	N	N
FSL R8-1305	Anatum	human, clinical	Y	N	N	N
FSL R8-1400	Anatum	environment, farm	Y	N	N	N
FSL R8-1401	Anatum	environment, farm	Y	N	N	N
FSL R8-1402	Anatum	environment, farm	Y	N	N	N
FSL R8-1404	Anatum	environment, farm	Y	N	N	N
FSL R8-1625	Anatum	environment, farm	Y	N	N	N
FSL R8-1819	anatum	animal, clinical	Y	N	N	N
FSL R8-1820	anatum	animal, clinical	Y	N	N	N
FSL R8-1853	anatum	animal, clinical	Y	N	N	N
FSL R8-1854	anatum	animal, clinical	Y	N	N	N
FSL R8-1855	anatum	animal, clinical	Y	N	N	N
FSL R8-1879	anatum	animal, clinical	Y	N	N	N
FSL R8-2108	Anatum	human, clinical	Y	N	N	N
FSL R8-3652	Anatum	environment, farm	Y	N	N	N
FSL R8-3653	Anatum	environment, farm	Y	N	N	N
FSL R8-4506	Anatum	human, clinical	Y	N	N	N
FSL R8-5396	Anatum	human, clinical	Y	N	N	N
FSL S5-0529	Anatum	human	Y	N	N	N
FSL S5-0530	Anatum	human	Y	N	N	N
FSL S5-0540	Anatum	human	Y	N	N	N
FSL R6-0929	Anatum var. 15+	environment, farm	Y	N	N	N
FSL R6-0930	Anatum var. 15+	environment, farm	Y	N	N	N
FSL R8-0347	Anatum var. 15+	environment, farm	Y	N	N	N
FSL R8-0348	Anatum var. 15+	environment, farm	Y	N	N	N
FSL R8-0851	Anatum var. 15+	animal, non-clinical	Y	N	N	N
FSL R8-0852	Anatum var. 15+	animal, non-clinical	Y	N	N	N
FSL R8-0853	Anatum var. 15+	animal, non-clinical	Y	N	N	N
FSL R8-0854	Anatum var. 15+	animal, non-clinical	Y	N	N	N
FSL A4-0677	Bareilly	human	Y	N	N	N
FSL R8-2449	Bareilly	human, clinical	Y	N	N	N
FSL R8-2587	Bareilly	human, clinical	Y	N	N	N
FSL R8-3741	Bareilly	human, clinical	Y	N	N	N
FSL R8-3886	Bareilly	human, clinical	Y	N	N	N
FSL R8-5210	Bareilly	human, clinical	Y	N	N	N
FSL R8-8142	Bareilly	human, clinical	Y	N	N	N
FSL R8-9535	Bareilly	human, clinical	Y	N	N	N
FSL R9-0050	Bareilly	human, clinical	Y	N	N	N
FSL R9-0059	Bareilly	human, clinical	Y	N	N	N
FSL R9-0061	Bareilly	human, clinical	Y	N	N	N
FSL R9-0099	Bareilly	human, clinical	Y	N	N	N
FSL R9-0144	Bareilly	human, clinical	Y	N	N	N
FSL R9-0146	Bareilly	human, clinical	Y	N	N	N
FSL R9-1723	Bareilly	human, clinical	Y	N	N	N
FSL M8-0474	Bareilly	poultry feather meal	Y	N	N	N
FSL M8-0475	Bareilly	feather meal	Y	N	N	N
FSL M8-0476	Bareilly	irrigation water	Y	N	N	N
FSL M8-0477	Bareilly	nonfat dry milk	Y	N	N	N
FSL M8-0478	Bareilly	whisker fish	Y	N	N	N
FSL M8-0479	Bareilly	fennel seeds	Y	N	N	N
FSL M8-0481	Bareilly	pepper, ground red	Y	N	N	N

FSL A4-0622	Berta	human	Y	N	N	N
FSL A4-0805	Berta	human	Y	N	N	N
FSL R8-1568	Berta	human, clinical	Y	N	N	N
FSL R8-1606	Berta	human, clinical	Y	N	N	N
FSL R8-2917	Berta	human, clinical	Y	N	N	N
FSL R8-3761	Berta	human, clinical	Y	N	N	N
FSL R8-4514	Berta	human, clinical	Y	N	N	N
FSL R8-4531	Berta	human, clinical	Y	N	N	N
FSL R8-4681	Berta	human, clinical	Y	N	N	N
FSL R8-5033	Berta	human, clinical	Y	N	N	N
FSL R8-5395	Berta	human, clinical	Y	N	N	N
FSL R8-6083	Berta	human, clinical	Y	N	N	N
FSL R8-8681	Berta	human	Y	N	N	N
FSL R8-9524	Berta	human, clinical	Y	N	N	N
FSL R8-9525	Berta	human, clinical	Y	N	N	N
FSL R8-9532	Berta	human, clinical	Y	N	N	N
FSL R9-0297	Berta	human, clinical	Y	N	N	N
FSL R9-1179	Berta	human, clinical	Y	N	N	N
FSL S5-0502	Berta	human	Y	N	N	N
FSL S5-0637	Berta	human	Y	N	N	N
FSL A4-0555	Braenderup	human	Y	N	N	N
FSL A4-0616	Braenderup	human	Y	N	N	N
FSL A4-0694	Braenderup	human	Y	N	N	N
FSL A4-0825	Braenderup	human	Y	N	N	N
FSL A4-0841	Braenderup	human	Y	N	N	N
FSL M8-0493	Braenderup	shrimp, frozen	Y	N	N	N
FSL R6-0213	Braenderup	human, clinical	Y	N	N	N
FSL R8-0286	Braenderup	human, clinical	Y	N	N	N
FSL R8-0461	Braenderup	human, clinical	Y	N	N	N
FSL R8-1284	Braenderup	human, clinical	Y	N	N	N
FSL R8-1293	Braenderup	human, clinical	Y	N	N	N
FSL R8-1533	Braenderup	human, clinical	Y	N	N	N
FSL R8-1567	Braenderup	human, clinical	Y	N	N	N
FSL R8-1576	Braenderup	human, clinical	Y	N	N	N
FSL R8-2517	Braenderup	human, clinical	Y	N	N	N
FSL R8-2544	Braenderup	human, clinical	Y	N	N	N
FSL R8-2922	Braenderup	human, clinical	Y	N	N	N
FSL R8-2944	Braenderup	human, clinical	Y	N	N	N
FSL R8-2950	Braenderup	human, clinical	Y	N	N	N
FSL R8-3373	Braenderup	human, clinical	Y	N	N	N
FSL R8-3503	Braenderup	human, clinical	Y	N	N	N
FSL R8-3549	Braenderup	human, clinical	Y	N	N	N
FSL R8-3676	Braenderup	human, clinical	Y	N	N	N
FSL R8-3678	Braenderup	human, clinical	Y	N	N	N
FSL R8-3733	Braenderup	human, clinical	Y	N	N	N
FSL R8-3883	Braenderup	human, clinical	Y	N	N	N
FSL R8-4415	Braenderup	human, clinical	Y	N	N	N
FSL R8-4460	Braenderup	human, clinical	Y	N	N	N
FSL R8-4547	Braenderup	human, clinical	Y	N	N	N
FSL R8-4658	Braenderup	human, clinical	Y	N	N	N
FSL R8-4849	Braenderup	animal, clinical	Y	N	N	N
FSL R8-4978	Braenderup	human, clinical	Y	N	N	N
FSL R8-5017	Braenderup	human, clinical	Y	N	N	N
FSL R8-5387	Braenderup	human, clinical	Y	N	N	N
FSL R8-6090	Braenderup	human, clinical	Y	N	N	N
FSL R8-6532	Braenderup	animal, clinical	Y	N	N	N
FSL R8-7966	Braenderup	human, clinical	Y	N	N	N
FSL R8-8054	Braenderup	human, clinical	Y	N	N	N
FSL R8-8058	Braenderup	human, clinical	Y	N	N	N
FSL R8-8062	Braenderup	human, clinical	Y	N	N	N
FSL R8-9595	Braenderup	animal, clinical	Y	N	N	N
FSL R8-9602	Braenderup	animal, clinical	Y	N	N	N

FSL R8-9668	Braenderup	human, clinical	Y	N	N	N
FSL R8-9677	Braenderup	human, clinical	Y	N	N	N
FSL R9-0052	Braenderup	human, clinical	Y	N	N	N
FSL R9-0097	Braenderup	human, clinical	Y	N	N	N
FSL R9-0151	Braenderup	human, clinical	Y	N	N	N
FSL S10-0939	Braenderup	environment, farm	Y	N	N	N
FSL S10-0947	Braenderup	environment, farm	Y	N	N	N
FSL S5-0373	Braenderup	human	Y	N	N	N
FSL F6-0967	Enteritidis	human, clinical	Y	N	N	N
FSL R6-0876	Enteritidis	human, clinical	Y	N	N	N
FSL R6-0880	Enteritidis	human, clinical	Y	N	N	N
FSL R8-0096	Enteritidis	human, clinical	Y	N	N	N
FSL R8-0099	Enteritidis	human, clinical	Y	N	N	N
FSL R8-0100	Enteritidis	human, clinical	Y	N	N	N
FSL R8-0102	Enteritidis	human, clinical	Y	N	N	N
FSL R8-0120	Enteritidis	human, clinical	Y	N	N	N
FSL R8-0121	Enteritidis	human, clinical	Y	N	N	N
FSL R8-0803	Enteritidis	human, clinical	Y	N	N	N
FSL R8-0804	Enteritidis	human, clinical	Y	N	N	N
FSL R8-0808	Enteritidis	human, clinical	Y	N	N	N
FSL R8-1577	Enteritidis	human, clinical	Y	N	N	N
FSL R8-1580	Enteritidis	human, clinical	Y	N	N	N
FSL R8-1581	Enteritidis	human, clinical	Y	N	N	N
FSL R8-1582	Enteritidis	human, clinical	Y	N	N	N
FSL R8-1587	Enteritidis	human, clinical	Y	N	N	N
FSL R8-1589	Enteritidis	human, clinical	Y	N	N	N
FSL R8-1601	Enteritidis	human, clinical	Y	N	N	N
FSL R8-1608	Enteritidis	human, clinical	Y	N	N	N
FSL R8-1609	Enteritidis	human, clinical	Y	N	N	N
FSL R8-3498	Enteritidis	human, clinical	Y	N	N	N
FSL R8-3500	Enteritidis	human, clinical	Y	N	N	N
FSL R8-3505	Enteritidis	human, clinical	Y	N	N	N
FSL R8-3532	Enteritidis	human, clinical	Y	N	N	N
FSL R8-3533	Enteritidis	human, clinical	Y	N	N	N
FSL R8-3534	Enteritidis	human, clinical	Y	N	N	N
FSL R8-3538	Enteritidis	human, clinical	Y	N	N	N
FSL R8-3539	Enteritidis	human, clinical	Y	N	N	N
FSL R8-3542	Enteritidis	human, clinical	Y	N	N	N
FSL R8-3543	Enteritidis	human, clinical	Y	N	N	N
FSL R8-3544	Enteritidis	human, clinical	Y	N	N	N
FSL R8-3548	Enteritidis	human, clinical	Y	N	N	N
FSL R8-3552	Enteritidis	human, clinical	Y	N	N	N
FSL R8-3553	Enteritidis	human, clinical	Y	N	N	N
FSL R8-3556	Enteritidis	human, clinical	Y	N	N	N
FSL R8-4074	Enteritidis	environment, farm	Y	N	N	N
FSL R8-4075	Enteritidis	animal, clinical	Y	N	N	N
FSL R8-4086	Enteritidis	environment, farm	Y	N	N	N
FSL S10-1621	Enteritidis	environment, farm	Y	N	N	N
FSL S10-1623	Enteritidis	environment, farm	Y	N	N	N
FSL S10-1644	Enteritidis	environment, farm	Y	N	N	N
FSL S10-1646	Enteritidis	environment, farm	Y	N	N	N
FSL S5-0271	Enteritidis	animal	Y	N	N	N
FSL S5-0279	Enteritidis	animal	Y	N	N	N
FSL S5-0280	Enteritidis	animal	Y	N	N	N
FSL S5-0281	Enteritidis	animal	Y	N	N	N
FSL S5-0282	Enteritidis	animal	Y	N	N	N
FSL S5-0283	Enteritidis	animal	Y	N	N	N
FSL S5-0284	Enteritidis	animal	Y	N	N	N
FSL R8-5224	I 13,23:b:-	type strain	Y	N	N	N
FSL A4-0563	Hadar	human	Y	N	N	N
FSL A4-0588	Hadar	human	Y	N	N	N
FSL A4-0621	Hadar	human	Y	N	N	N

FSL A4-0681	Hadar	human	Y	N	N	N
FSL A4-0698	Hadar	human	Y	N	N	N
FSL A4-0836	Hadar	human	Y	N	N	N
FSL M8-0508	Hadar	Turkey, raw	Y	N	N	N
FSL M8-0520	Hadar	Turkey carcass swab	Y	N	N	N
FSL M8-0521	Hadar	Young chicken rinse	Y	N	N	N
FSL M8-0522	Hadar	Ground beef	Y	N	N	N
FSL M8-0523	Hadar	Ground turkey	Y	N	N	N
FSL R6-0306	Hadar	human, clinical	Y	N	N	N
FSL R6-0394	Hadar	animal, clinical	Y	N	N	N
FSL R6-0515	Hadar	animal, clinical	Y	N	N	N
FSL R8-0117	Hadar	human, clinical	Y	N	N	N
FSL R8-1289	Hadar	human, clinical	Y	N	N	N
FSL R8-1610	Hadar	human, clinical	Y	N	N	N
FSL R8-2109	Hadar	human, clinical	Y	N	N	N
FSL R8-2529	Hadar	human, clinical	Y	N	N	N
FSL R8-2538	Hadar	human, clinical	Y	N	N	N
FSL R8-2558	Hadar	human, clinical	Y	N	N	N
FSL R8-2603	Hadar	human, clinical	Y	N	N	N
FSL R8-2609	Hadar	human, clinical	Y	N	N	N
FSL R8-2614	Hadar	human, clinical	Y	N	N	N
FSL R8-2780	Hadar	human, clinical	Y	N	N	N
FSL R8-2782	Hadar	human, clinical	Y	N	N	N
FSL R8-2783	Hadar	human, clinical	Y	N	N	N
FSL R8-2784	Hadar	human, clinical	Y	N	N	N
FSL R8-2920	Hadar	human, clinical	Y	N	N	N
FSL R8-2990	Hadar	human, clinical	Y	N	N	N
FSL R8-2991	Hadar	human, clinical	Y	N	N	N
FSL R8-3392	Hadar	human, clinical	Y	N	N	N
FSL R8-3509	Hadar	human, clinical	Y	N	N	N
FSL R8-3566	Hadar	human, clinical	Y	N	N	N
FSL R8-3644	Hadar	human, clinical	Y	N	N	N
FSL R8-3708	Hadar	human, clinical	Y	N	N	N
FSL R8-4060	Hadar	human, clinical	Y	N	N	N
FSL R8-4436	Hadar	human, clinical	Y	N	N	N
FSL R8-4466	Hadar	human, clinical	Y	N	N	N
FSL R8-4557	Hadar	human, clinical	Y	N	N	N
FSL R8-5219	Hadar	human, clinical	Y	N	N	N
FSL R8-5933	Hadar	animal, clinical	Y	N	N	N
FSL R8-5935	Hadar	animal, clinical	Y	N	N	N
FSL R8-6091	Hadar	human, clinical	Y	N	N	N
FSL R9-1719	Hadar	human, clinical	Y	N	N	N
FSL S5-0399	Hadar	human	Y	N	N	N
FSL S5-0543	Hadar	human	Y	N	N	N
FSL A4-0591	Hartford	human	Y	N	Y[a]	N
FSL A4-0600	Hartford	human	Y	N	N	N
FSL A4-0617	Hartford	human	Y	N	N	N
FSL M8-0492	Hartford	raw oysters	Y	N	N	N
FSL M8-0518	Hartford	human, clinical	Y	N	N	N
FSL R6-0220	Hartford	human, clinical	Y	N	N	N
FSL R6-0371	Hartford	animal, clinical	Y	N	N	N
FSL R6-0620	Hartford	animal, clinical	Y	N	Y[a]	N
FSL R8-2102	Hartford	human, clinical	Y	N	N	N
FSL R8-2478	Hartford	human, clinical	Y	N	N	N
FSL R8-2493	Hartford	human, clinical	Y	N	N	N
FSL R8-4058	Hartford	human, clinical	Y	N	N	N
FSL R8-4430	Hartford	human, clinical	Y	N	N	N
FSL R8-4521	Hartford	human, clinical	Y	N	N	N
FSL R8-8628	Hartford	animal, clinical	Y	N	N	N
FSL R8-9607	Hartford	animal, clinical	Y	N	N	N
FSL R9-0034	Hartford	human, clinical	Y	N	N	N
FSL S5-0510	Hartford	human	Y	N	Y[a]	N

FSL R9-0453	Heidelberg	human, clinical	Y	N	N	N
FSL R6-0205	Heidelberg	human, clinical	Y	N	N	N
FSL R6-0210	Heidelberg	human, clinical	Y	N	N	N
FSL R6-0314	Heidelberg	human, clinical	Y	N	N	N
FSL R6-0529	Heidelberg	human	Y	N	N	N
FSL R6-0537	Heidelberg	human	Y	N	N	N
FSL R6-0886	Heidelberg	human, clinical	Y	N	N	N
FSL R6-0988	Heidelberg	human, clinical	Y	N	N	N
FSL R6-0991	Heidelberg	human, clinical	Y	N	N	N
FSL R8-0106	Heidelberg	human, clinical	Y	N	N	N
FSL R8-0109	Heidelberg	human, clinical	Y	N	N	N
FSL R8-0145	Heidelberg	human, clinical	Y	N	N	N
FSL R8-0284	Heidelberg	human, clinical	Y	N	N	N
FSL R8-0463	Heidelberg	human, clinical	Y	N	N	N
FSL R8-2241	Heidelberg	environment, farm	Y	N	N	N
FSL R8-3508	Heidelberg	human, clinical	Y	N	N	N
FSL R8-3547	Heidelberg	human, clinical	Y	N	N	N
FSL R8-3571	Heidelberg	human, clinical	Y	N	N	N
FSL R8-3573	Heidelberg	human, clinical	Y	N	N	N
FSL R8-3705	Heidelberg	human, clinical	Y	N	N	N
FSL R8-3852	Heidelberg	animal, clinical	Y	N	N	N
FSL R8-4446	Heidelberg	human, clinical	Y	N	N	N
FSL R8-8014	Heidelberg	animal, clinical	Y	N	N	N
FSL R8-8015	Heidelberg	animal, clinical	Y	N	N	N
FSL R8-8016	Heidelberg	animal, clinical	Y	N	N	N
FSL R8-8018	Heidelberg	animal, clinical	Y	N	N	N
FSL R8-8022	Heidelberg	animal, clinical	Y	N	N	N
FSL R8-8023	Heidelberg	animal, clinical	Y	N	N	N
FSL R8-8392	Heidelberg	animal, clinical	Y	N	N	N
FSL R8-9089	Heidelberg	animal, clinical	Y	N	N	N
FSL R8-9129	Heidelberg	animal, clinical	Y	N	N	N
FSL R8-9800	Heidelberg	animal, clinical	Y	N	N	N
FSL R9-0300	Heidelberg	human, clinical	Y	N	N	N
FSL R9-0451	Heidelberg	human, clinical	Y	N	N	N
FSL R9-0983	Heidelberg	human, clinical	Y	N	N	N
FSL R9-1082	Heidelberg	animal, clinical	Y	N	N	N
FSL R9-1263	Heidelberg	animal, clinical	Y	N	N	N
FSL R9-1393	Heidelberg	human, clinical	Y	N	N	N
FSL S3-0901	Heidelberg	animal	Y	N	N	N
FSL S3-0902	Heidelberg	animal	Y	N	N	N
FSL S3-0919	Heidelberg	animal	Y	N	N	N
FSL S3-0920	Heidelberg	animal	Y	N	N	N
FSL S3-0921	Heidelberg	animal	Y	N	N	N
FSL S5-0448	Heidelberg	human	Y	N	N	N
FSL S5-0455	Heidelberg	human	Y	N	N	N
FSL S5-0475	Heidelberg	human	Y	N	N	N
FSL S5-0480	Heidelberg	human	Y	N	N	N
FSL S5-0491	Heidelberg	human	Y	N	N	N
FSL S5-0495	Heidelberg	human	Y	N	N	N
FSL S5-0655	Heidelberg	human	Y	N	N	N
FSL R8-2121	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R8-2602	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R8-9523	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R8-9533	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R9-0090	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R9-0150	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R9-0298	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R9-0301	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R8-3683	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R8-3502	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R8-3369	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R8-3672	I 4, 5,12:i:-	human, clinical	Y	N	N	N

FSL R8-4066	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R8-3504	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R8-1299	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R8-1300	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R8-1310	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R8-1584	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R8-1586	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R8-1604	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R8-1620	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R8-2104	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R8-2532	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R8-2930	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R8-3399	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R8-3523	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R8-3546	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R8-3707	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R8-4391	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R8-4442	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R8-4504	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R8-4508	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R8-4536	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R8-4549	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R8-4972	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R8-6088	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R8-6459	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R8-8056	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R8-8074	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R9-0464	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R9-0835	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R9-0840	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R9-1180	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R9-1259	I 4, 5,12:i:-	animal, clinical	Y	N	N	N
FSL R9-1273	I 4, 5,12:i:-	animal, clinical	Y	N	N	N
FSL R9-1447	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R9-1727	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL R9-1728	I 4, 5,12:i:-	human, clinical	Y	N	N	N
FSL S10-1237	I 4, 5,12:i:-	environment, farm	Y	N	N	N
FSL S10-1241	I 4, 5,12:i:-	environment, farm	Y	N	N	N
FSL A4-0546	Infantis	animal	Y	N	N	N
FSL R6-0206	Infantis	human, clinical	Y	N	N	N
FSL R6-0789	Infantis	environment, farm	Y	N	N	N
FSL R6-0790	Infantis	environment, farm	Y	N	N	N
FSL R6-0791	Infantis	environment, farm	Y	N	N	N
FSL R6-0931	Infantis	environment, farm	Y	N	N	N
FSL R6-0932	Infantis	environment, farm	Y	N	N	N
FSL R8-0452	Infantis	human, clinical	Y	N	N	N
FSL R8-0770	Infantis	human, clinical	Y	N	N	N
FSL R8-0801	Infantis	human, clinical	Y	N	N	N
FSL R8-1392	Infantis	environment, farm	Y	N	N	N
FSL R8-1569	Infantis	human, clinical	Y	N	N	N
FSL R8-2479	Infantis	human, clinical	Y	N	N	N
FSL R8-2525	Infantis	human, clinical	Y	N	N	N
FSL R8-2598	Infantis	human, clinical	Y	N	N	N
FSL R8-2601	Infantis	human, clinical	Y	N	N	N
FSL R8-2811	Infantis	environment, farm	Y	N	N	N
FSL R8-2973	Infantis	human, clinical	Y	N	N	N
FSL R8-3391	Infantis	human, clinical	Y	N	N	N
FSL R8-8053	Infantis	human, clinical	Y	N	N	N
FSL R8-8080	Infantis	human, clinical	Y	N	N	N
FSL R8-8691	Infantis	human, clinical	Y	N	N	N
FSL R8-9662	Infantis	human, clinical	Y	N	N	N
FSL R8-9667	Infantis	human, clinical	Y	N	N	N

FSL R9-0149	Infantis	human, clinical	Y	N	N	N
FSL R9-0987	Infantis	human, clinical	Y	N	N	N
FSL R9-1070	Infantis	environment, farm	Y	N	N	N
FSL S10-1647	Infantis	environment, farm	Y	N	N	N
FSL S10-1665	Infantis	environment, farm	Y	N	N	N
FSL S10-1666	Infantis	environment, farm	Y	N	N	N
FSL S10-1669	Infantis	environment, farm	Y	N	N	N
FSL S10-1670	Infantis	environment, farm	Y	N	N	N
FSL S5-0506	Infantis	human	Y	N	N	N
FSL S5-0553	Infantis	animal	Y	N	N	N
FSL S5-0560	Infantis	animal	Y	N	N	N
FSL S5-0566	Infantis	animal	Y	N	N	N
FSL S5-0571	Infantis	animal	Y	N	N	N
FSL S5-0572	Infantis	animal	Y	N	N	N
FSL S5-0573	Infantis	animal	Y	N	N	N
FSL S5-0574	Infantis	animal	Y	N	N	N
FSL S5-0575	Infantis	animal	Y	N	N	N
FSL S5-0576	Infantis	animal	Y	N	N	N
FSL S5-0582	Infantis	animal	Y	N	N	N
FSL S5-0584	Infantis	animal	Y	N	N	N
FSL S5-0593	Infantis	animal	Y	N	N	N
FSL S5-0617	Infantis	animal	Y	N	N	N
FSL S5-0725	Infantis	animal	Y	N	N	N
FSL S5-0734	Infantis	animal	Y	N	N	N
FSL S5-0743	Infantis	animal	Y	N	N	N
FSL S5-0750	Infantis	animal	Y	N	N	N
FSL A4-0574	Javiana	human	Y	Y	Y	Y
FSL A4-0602	Javiana	human	Y	Y	Y	Y
FSL A4-0613	Javiana	human	Y	Y	Y	Y
FSL A4-0679	Javiana	human	Y	Y	Y	Y
FSL A4-0690	Javiana	human	Y	Y	Y	Y
FSL A4-0696	Javiana	human	Y	Y	Y	Y
FSL M8-0480	Javiana	creek sediment	Y	Y	Y	Y
FSL M8-0489	Javiana	creek sediment	Y	Y	Y	Y
FSL M8-0491	Javiana	creek water	Y	Y	Y	Y
FSL M8-0494	Javiana	canal water	Y	Y	Y	Y
FSL M8-0495	Javiana	stream water	Y	Y	Y	Y
FSL M8-0496	Javiana	Basil	Y	Y	Y	Y
FSL M8-0497	Javiana	Frog Legs	Y	Y	Y	Y
FSL M8-0498	Javiana	Cantaloupe	Y	Y	Y	Y
FSL M8-0510	Javiana	Cheese	Y	Y	Y	Y
FSL R6-0243	Javiana	human	Y	Y	Y	Y
FSL R6-0248	Javiana	human	Y	Y	Y	Y
FSL R6-0253	Javiana	human	Y	Y	Y	Y
FSL R6-0312	Javiana	human, clinical	Y	Y	Y	Y
FSL R6-0533	Javiana	human	Y	Y	Y	Y
FSL R6-0985	Javiana	human, clinical	Y	Y	Y	Y
FSL R8-0293	Javiana	human, clinical	Y	Y	Y	Y
FSL R8-0301	Javiana	human, clinical	Y	Y	Y	Y
FSL R8-2462	Javiana	human, clinical	Y	Y	Y	Y
FSL R8-2516	Javiana	human, clinical	Y	Y	Y	Y
FSL R8-2919	Javiana	human, clinical	Y	Y	Y	Y
FSL R8-2936	Javiana	human, clinical	Y	Y	Y	Y
FSL R8-2972	Javiana	human, clinical	Y	Y	Y	Y
FSL R8-3888	Javiana	human, clinical	Y	Y	Y	Y
FSL R8-4395	Javiana	human, clinical	Y	Y	Y	Y
FSL R8-4530	Javiana	human, clinical	Y	Y	Y	Y
FSL R8-4534	Javiana	human, clinical	Y	Y	Y	Y
FSL R8-4543	Javiana	human, clinical	Y	Y	Y	Y
FSL R8-4562	Javiana	human, clinical	Y	Y	Y	Y
FSL R8-4655	Javiana	human, clinical	Y	Y	Y	Y
FSL R8-4958	Javiana	human, clinical	Y	Y	Y	Y

FSL R8-5214	Javiana	human, clinical	Y	Y	Y	Y
FSL R8-6103	Javiana	human, clinical	Y	Y	Y	Y
FSL R9-2204	Javiana	animal, clinical	Y	Y	Y	Y
FSL S5-0290	Javiana	animal	Y	Y	Y	Y
FSL S5-0359	Javiana	human	Y	Y	Y	Y
FSL S5-0360	Javiana	human	Y	Y	Y	Y
FSL S5-0361	Javiana	human	Y	Y	Y	Y
FSL S5-0362	Javiana	human	Y	Y	Y	Y
FSL S5-0363	Javiana	human	Y	Y	Y	Y
FSL S5-0395	Javiana	human	Y	Y	Y	Y
FSL S5-0406	Javiana	human	Y	Y	Y	Y
FSL S5-0652	Javiana	human	Y	Y	Y	Y
FSL S5-0665	Javiana	human	Y	Y	Y	Y
FSL S5-0675	Javiana	human	Y	Y	Y	Y
FSL A4-0633	Mississippi	human	Y	N	N	N
FSL M8-0515	Mississippi	Food	Y	N	N	N
FSL M8-0516	Mississippi	Food	Y	N	N	N
FSL R8-1549	Mississippi	human, clinical	Y	Y	Y	Y
FSL R8-2455	Mississippi	human, clinical	Y	Y	Y	Y
FSL R8-2593	Mississippi	human, clinical	Y	N	N	N
FSL R8-2779	Mississippi	human, clinical	Y	Y	Y	Y
FSL R8-7996	Mississippi	human, clinical	Y	Y	Y	Y
FSL A4-0562	Montevideo	human	Y	Y	Y	Y
FSL R6-0873	Montevideo	human, clinical	Y	Y	Y	Y
FSL R6-0884	Montevideo	human, clinical	Y	Y	Y	Y
FSL R8-0129	Montevideo	human, clinical	Y	Y	Y	Y
FSL R8-0147	Montevideo	human, clinical	Y	Y	Y	Y
FSL R8-1540	Montevideo	human, clinical	Y	Y	Y	Y
FSL R8-1552	Montevideo	human, clinical	Y	Y	Y	Y
FSL R8-1602	Montevideo	human, clinical	Y	Y	Y	Y
FSL R8-1613	Montevideo	human, clinical	Y	Y	Y	Y
FSL R8-2004	Montevideo	environment, farm	Y	Y	Y	Y
FSL R8-2005	Montevideo	environment, farm	Y	Y	Y	Y
FSL R8-2106	Montevideo	human, clinical	Y	Y	Y	Y
FSL R8-2165	Montevideo	animal, clinical	Y	Y	Y	Y
FSL R8-2533	Montevideo	human, clinical	Y	Y	Y	Y
FSL R8-2589	Montevideo	human, clinical	Y	Y	Y	Y
FSL R8-2607	Montevideo	human, clinical	Y	Y	Y	Y
FSL R8-2812	Montevideo	environment, farm	Y	Y	Y	Y
FSL R8-2849	Montevideo	environment, farm	Y	Y	Y	Y
FSL R8-2868	Montevideo	animal, non-clinical	Y	Y	Y	Y
FSL R8-2923	Montevideo	human, clinical	Y	Y	Y	Y
FSL R8-3400	Montevideo	human, clinical	Y	Y	Y	Y
FSL R8-3417	Montevideo	environment, farm	Y	Y	Y	Y
FSL R8-3559	Montevideo	human, clinical	Y	Y	Y	Y
FSL R8-3658	Montevideo	environment, farm	Y	Y	Y	Y
FSL R8-3659	Montevideo	environment, farm	Y	Y	Y	Y
FSL R8-3854	Montevideo	animal, clinical	Y	Y	Y	Y
FSL R8-4841	Montevideo	human, clinical	Y	Y	Y	Y
FSL R8-4917	Montevideo	animal, clinical	Y	Y	Y	Y
FSL R8-4918	Montevideo	human, clinical	Y	Y	Y	Y
FSL R8-4919	Montevideo	human, clinical	Y	Y	Y	Y
FSL R8-4920	Montevideo	human, clinical	Y	Y	Y	Y
FSL R8-4921	Montevideo	human, clinical	Y	Y	Y	Y
FSL R8-4922	Montevideo	human, clinical	Y	Y	Y	Y
FSL R8-4923	Montevideo	human, clinical	Y	Y	Y	Y
FSL R8-7305	Montevideo	animal, clinical	Y	Y	Y	Y
FSL R9-1588	Montevideo	environment, farm	Y	Y	Y	Y
FSL S5-0264	Montevideo	animal	Y	Y	Y	Y
FSL S5-0265	Montevideo	animal	Y	Y	Y	Y
FSL S5-0266	Montevideo	animal	Y	Y	Y	Y
FSL S5-0382	Montevideo	human	Y	Y	Y	Y

FSL S5-0457	Montevideo	human	Y	Y	Y	Y
FSL S5-0463	Montevideo	human	Y	Y	Y	Y
FSL S5-0470	Montevideo	human	Y	Y	Y	Y
FSL S5-0474	Montevideo	human	Y	Y	Y	Y
FSL S5-0478	Montevideo	human	Y	Y	Y	Y
FSL S5-0559	Montevideo	animal	Y	Y	Y	Y
FSL S5-0630	Montevideo	animal	Y	Y	Y	Y
FSL S5-0757	Montevideo	animal	Y	Y	Y	Y
FSL A4-0649	Muenchen	human	Y	N	N	N
FSL M8-0509	Muenchen	Frog Legs	Y	N	N	N
FSL M8-0511	Muenchen	Orange Juice	Y	N	N	N
FSL M8-0512	Muenchen	Sea Water	Y	N	N	N
FSL M8-0513	Muenchen	Nonfat Dry Milk	Y	N	N	N
FSL M8-0514	Muenchen	Dairy Environment	Y	N	N	N
FSL M8-0517	Muenchen	Raw Oysters	Y	N	N	N
FSL R6-0093	Muenchen	animal	Y	N	N	N
FSL R6-0309	Muenchen	human, clinical	Y	N	N	N
FSL R6-0881	Muenchen	human, clinical	Y	N	N	N
FSL R8-0149	Muenchen	human, clinical	Y	N	N	N
FSL R8-0777	Muenchen	human, clinical	Y	N	N	N
FSL R8-1592	Muenchen	human, clinical	Y	N	N	N
FSL R8-1596	Muenchen	human, clinical	Y	N	N	N
FSL R8-1845	muenchen	animal, clinical	Y	N	N	N
FSL R8-3288	muenchen	animal, clinical	Y	N	N	N
FSL R8-3506	Muenchen	human, clinical	Y	N	N	N
FSL R8-3550	Muenchen	human, clinical	Y	N	N	N
FSL R8-4059	Muenchen	human, clinical	Y	N	N	N
FSL R8-4399	Muenchen	human, clinical	Y	N	N	N
FSL R8-4400	Muenchen	human, clinical	Y	N	N	N
FSL R8-4401	Muenchen	human, clinical	Y	N	N	N
FSL R8-4407	Muenchen	human, clinical	Y	N	N	N
FSL R8-4440	Muenchen	human, clinical	Y	N	N	N
FSL R8-4463	Muenchen	human, clinical	Y	N	N	N
FSL R8-4833	Muenchen	animal, clinical	Y	N	N	N
FSL R8-4850	Muenchen	animal, clinical	Y	N	N	N
FSL R8-4858	Muenchen	animal, clinical	Y	N	N	N
FSL R8-6093	Muenchen	human, clinical	Y	N	N	N
FSL R8-8211	Muenchen	animal, clinical	Y	N	N	N
FSL R8-8227	Muenchen	animal, clinical	Y	N	N	N
FSL R8-8228	Muenchen	animal, clinical	Y	N	N	N
FSL R8-8229	Muenchen	animal, clinical	Y	N	N	N
FSL R8-8235	Muenchen	animal, clinical	Y	N	N	N
FSL R8-8236	Muenchen	animal, clinical	Y	N	N	N
FSL R8-8239	Muenchen	animal, clinical	Y	N	N	N
FSL R8-8240	Muenchen	animal, clinical	Y	N	N	N
FSL R8-8375	Muenchen	animal, clinical	Y	N	N	N
FSL R8-8689	Muenchen	human, clinical	Y	N	N	N
FSL R8-9195	Muenchen	human, clinical	Y	N	N	N
FSL R8-9624	Muenchen	animal, clinical	Y	N	N	N
FSL R8-9634	Muenchen	animal, clinical	Y	N	N	N
FSL R8-9844	Muenchen	animal, clinical	Y	N	N	N
FSL R9-0041	Muenchen	human, clinical	Y	N	N	N
FSL R9-1108	Muenchen	animal, clinical	Y	N	N	N
FSL S5-0479	Muenchen	human	Y	N	N	N
FSL S5-0504	Muenchen	human	Y	N	N	N
FSL S5-0636	Muenchen	human	Y	N	N	N
FSL R6-0001	Newport	animal	Y	N	N	N
FSL R6-0004	Newport	animal	Y	N	N	N
FSL R6-0005	Newport	animal	Y	N	N	N
FSL R6-0007	Newport	animal	Y	N	N	N
FSL R6-0010	Newport	animal	Y	N	N	N
FSL R6-0011	Newport	animal	Y	N	N	N

FSL R6-0204	Newport	human, clinical	Y	N	N	N
FSL R6-0218	Newport	human, clinical	Y	N	N	N
FSL R8-0341	Newport	environment, farm	Y	N	N	N
FSL R8-0342	Newport	environment, farm	Y	N	N	N
FSL R8-1117	Newport	human, clinical	Y	N	N	N
FSL R8-1118	Newport	human, clinical	Y	N	N	N
FSL R8-1119	Newport	human, clinical	Y	N	N	N
FSL R8-1120	Newport	human, clinical	Y	N	N	N
FSL R8-1121	Newport	human, clinical	Y	N	N	N
FSL R8-1637	Newport	environment, farm	Y	N	N	N
FSL R8-1638	Newport	environment, farm	Y	N	N	N
FSL R8-1639	Newport	environment, farm	Y	N	N	N
FSL R8-2248	Newport	environment, farm	Y	N	N	N
FSL R8-2350	newport	animal, clinical	Y	N	N	N
FSL R8-2351	newport	animal, clinical	Y	N	N	N
FSL R8-2353	newport	animal, clinical	Y	N	N	N
FSL R8-2490	Newport	human, clinical	Y	N	N	N
FSL R8-2512	Newport	human, clinical	Y	N	N	N
FSL R8-3908	Newport	animal, clinical	Y	N	N	N
FSL R8-4085	Newport	environment, farm	Y	N	N	N
FSL R8-4159	Newport	animal, clinical	Y	N	N	N
FSL R8-4315	Newport	animal, clinical	Y	N	N	N
FSL R8-7260	Newport	animal, clinical	Y	N	N	N
FSL R8-8684	Newport	human, clinical	Y	N	N	N
FSL R8-8686	Newport	human, clinical	Y	N	N	N
FSL R8-9176	Newport	human, clinical	Y	N	N	N
FSL R8-9190	Newport	human, clinical	Y	N	N	N
FSL R8-9198	Newport	human, clinical	Y	N	N	N
FSL S10-1020	Newport	environment, farm	Y	N	N	N
FSL S10-1570	Newport	environment, farm	Y	N	N	N
FSL S10-1630	Newport	environment, farm	Y	N	N	N
FSL S10-1632	Newport	environment, farm	Y	N	N	N
FSL S10-1634	Newport	environment, farm	Y	N	N	N
FSL S10-1638	Newport	environment, farm	Y	N	N	N
FSL S10-1640	Newport	environment, farm	Y	N	N	N
FSL S10-1642	Newport	environment, farm	Y	N	N	N
FSL S10-1743	Newport	environment, farm	Y	N	N	N
FSL S10-1745	Newport	environment, farm	Y	N	N	N
FSL S10-1748	Newport	environment, farm	Y	N	N	N
FSL S10-1752	Newport	environment, farm	Y	N	N	N
FSL S10-1755	Newport	environment, farm	Y	N	N	N
FSL S10-1813	Newport	environment, farm	Y	N	N	N
FSL S5-0270	Newport	animal	Y	N	N	N
FSL S5-0272	Newport	animal	Y	N	N	N
FSL A4-0549	Oranienburg	animal	Y	Y	Y	Y
FSL A4-0564	Oranienburg	human	Y	Y	Y	Y
FSL A4-0594	Oranienburg	human	Y	Y	Y	Y
FSL A4-0642	Oranienburg	human	Y	Y	Y	Y
FSL A4-0644	Oranienburg	human	Y	Y	Y	Y
FSL A4-0668	Oranienburg	human	Y	Y	Y	Y
FSL A4-0688	Oranienburg	human	Y	Y	Y	Y
FSL A4-0809	Oranienburg	human	Y	Y	Y	Y
FSL R6-0209	Oranienburg	human, clinical	Y	Y	Y	Y
FSL R6-0240	Oranienburg	human	Y	Y	Y	Y
FSL R6-0586	Oranienburg	animal	Y	Y	Y	Y
FSL R6-0587	Oranienburg	animal, clinical	Y	Y	Y	Y
FSL R6-0594	Oranienburg	animal, clinical	Y	Y	Y	Y
FSL R6-0595	Oranienburg	animal, clinical	Y	Y	Y	Y
FSL R6-0598	Oranienburg	animal, clinical	Y	Y	Y	Y
FSL R8-0169	Oranienburg	environment, farm	Y	Y	Y	Y
FSL R8-0170	Oranienburg	environment, farm	Y	Y	Y	Y
FSL R8-1257	oranienburg	animal, clinical	Y	Y	Y	Y

FSL R8-1278	oranienburg	animal, clinical	Y	Y	Y	Y
FSL R8-1416	Oranienburg	environment, farm	Y	Y	Y	Y
FSL R8-1417	Oranienburg	environment, farm	Y	Y	Y	Y
FSL R8-1809	Oranienburg	animal, clinical	Y	Y	Y	Y
FSL R8-1963	Oranienburg	animal, clinical	Y	Y	Y	Y
FSL R8-2013	Oranienburg	environment, farm	Y	Y	Y	Y
FSL R8-2352	Oranienburg	animal, clinical	Y	Y	Y	Y
FSL R8-3270	Oranienburg	animal, clinical	Y	Y	Y	Y
FSL R8-3769	Oranienburg	human, clinical	Y	Y	Y	Y
FSL R8-3835	Oranienburg	animal, clinical	Y	Y	Y	Y
FSL R8-3884	Oranienburg	human, clinical	Y	Y	Y	Y
FSL R8-4067	Oranienburg	human, clinical	Y	Y	Y	Y
FSL R8-4427	Oranienburg	human, clinical	Y	Y	Y	Y
FSL R8-4439	Oranienburg	human, clinical	Y	Y	Y	Y
FSL R8-4443	Oranienburg	human, clinical	Y	Y	Y	Y
FSL R8-4532	Oranienburg	human, clinical	Y	Y	Y	Y
FSL R8-4662	Oranienburg	human, clinical	Y	Y	Y	Y
FSL R8-5000	Oranienburg	human, clinical	Y	Y	Y	Y
FSL R8-5030	Oranienburg	human, clinical	Y	Y	Y	Y
FSL R8-5060	Oranienburg	human, clinical	Y	Y	Y	Y
FSL R8-5072	Oranienburg	human, clinical	Y	Y	Y	Y
FSL R8-6094	Oranienburg	human, clinical	Y	Y	Y	Y
FSL R8-6542	Oranienburg	animal, clinical	Y	Y	Y	Y
FSL R8-7954	Oranienburg	human, clinical	Y	Y	Y	Y
FSL R8-8138	Oranienburg	human, clinical	Y	Y	Y	Y
FSL R8-8683	Oranienburg	human, clinical	Y	Y	Y	Y
FSL R8-9673	Oranienburg	human, clinical	Y	Y	Y	Y
FSL R9-0033	Oranienburg	human, clinical	Y	Y	Y	Y
FSL R9-0442	Oranienburg	human, clinical	Y	Y	Y	Y
FSL R9-1721	Oranienburg	human, clinical	Y	Y	Y	Y
FSL S5-0427	Oranienburg	animal	Y	Y	Y	Y
FSL S5-0642	Oranienburg	human	Y	Y	Y	Y
FSL A4-0634	Saintpaul	human	Y	N	N	N
FSL A4-0662	Saintpaul	human	Y	N	N	N
FSL A4-0687	Saintpaul	human	Y	N	N	N
FSL A4-0828	Saintpaul	human	Y	N	N	N
FSL M8-0499	Saintpaul	food	Y	N	N	N
FSL M8-0500	Saintpaul	environmental	Y	N	N	N
FSL M8-0501	Saintpaul	food	Y	N	N	N
FSL M8-0502	Saintpaul	environmental	Y	N	N	N
FSL M8-0503	Saintpaul	environmental	Y	N	N	N
FSL M8-0504	Saintpaul	environmental	Y	N	N	N
FSL M8-0505	Saintpaul	food	Y	N	N	N
FSL M8-0506	Saintpaul	food	Y	N	N	N
FSL M8-0507	Saintpaul	environmental	Y	N	N	N
FSL R6-0208	Saintpaul	human, clinical	Y	N	N	N
FSL R6-0219	Saintpaul	human, clinical	Y	N	N	N
FSL R6-0222	Saintpaul	human, clinical	Y	N	N	N
FSL R6-0399	Saintpaul	human, clinical	Y	N	N	N
FSL R6-0536	Saintpaul	human	Y	N	N	N
FSL R6-0990	Saintpaul	human, clinical	Y	N	N	N
FSL R8-1523	Saintpaul	human, clinical	Y	N	N	N
FSL R8-1531	Saintpaul	human, clinical	Y	N	N	N
FSL R8-1535	Saintpaul	human, clinical	Y	N	N	N
FSL R8-1585	Saintpaul	human, clinical	Y	N	N	N
FSL R8-2507	Saintpaul	human, clinical	Y	N	N	N
FSL R8-3582	Saintpaul	human, clinical	Y	N	N	N
FSL R8-3635	Saintpaul	human, clinical	Y	N	N	N
FSL R8-3641	Saintpaul	human, clinical	Y	N	N	N
FSL R8-3680	Saintpaul	human, clinical	Y	N	N	N
FSL R8-4063	Saintpaul	human, clinical	Y	N	N	N
FSL R8-4437	Saintpaul	human, clinical	Y	N	N	N

FSL R8-4445	Saintpaul	human, clinical	Y	N	N	N
FSL R8-4484	Saintpaul	human, clinical	Y	N	N	N
FSL R8-4652	Saintpaul	human, clinical	Y	N	N	N
FSL R8-4660	Saintpaul	human, clinical	Y	N	N	N
FSL R8-4670	Saintpaul	human, clinical	Y	N	N	N
FSL R8-4809	Saintpaul	human, clinical	Y	N	N	N
FSL R8-4907	Saintpaul	human, clinical	Y	N	N	N
FSL R8-4985	Saintpaul	human, clinical	Y	N	N	N
FSL R8-4992	Saintpaul	human, clinical	Y	N	N	N
FSL R8-4996	Saintpaul	human, clinical	Y	N	N	N
FSL R8-5023	Saintpaul	human, clinical	Y	N	N	N
FSL R8-5029	Saintpaul	human, clinical	Y	N	N	N
FSL R8-5058	Saintpaul	human, clinical	Y	N	N	N
FSL R8-6469	Saintpaul	human, clinical	Y	N	N	N
FSL R8-6472	Saintpaul	human, clinical	Y	N	N	N
FSL R8-7964	Saintpaul	human, clinical	Y	N	N	N
FSL R8-7989	Saintpaul	human, clinical	Y	N	N	N
FSL R8-8692	Saintpaul	human, clinical	Y	N	N	N
FSL R8-8946	Saintpaul	human, clinical	Y	N	N	N
FSL M8-0519	Saintpaul	food	Y	N	N	N
FSL P3-1113	Thompson	environment, farm	Y	N	N	N
FSL R6-0200	Thompson	human, clinical	Y	N	N	N
FSL R6-0202	Thompson	human, clinical	Y	N	N	N
FSL R6-0216	Thompson	human, clinical	Y	N	N	N
FSL R6-0217	Thompson	human, clinical	Y	N	N	N
FSL R6-0223	Thompson	human, clinical	Y	N	N	N
FSL R6-0225	Thompson	human, clinical	Y	N	N	N
FSL R8-0014	Thompson	human, clinical	Y	N	N	N
FSL R8-0123	Thompson	human, clinical	Y	N	N	N
FSL R8-0131	Thompson	human, clinical	Y	N	N	N
FSL R8-0152	Thompson	human, clinical	Y	N	N	N
FSL R8-1835	thompson	animal, clinical	Y	N	N	N
FSL R8-1846	thompson	animal, clinical	Y	N	N	N
FSL R8-3268	thompson	animal, clinical	Y	N	N	N
FSL R8-3269	thompson	animal, clinical	Y	N	N	N
FSL R8-3845	Thompson	animal, clinical	Y	N	N	N
FSL R8-4006	Thompson	animal, clinical	Y	N	N	N
FSL R8-4047	Thompson	animal, clinical	Y	N	N	N
FSL R8-4101	Thompson	animal, clinical	Y	N	N	N
FSL R8-4314	Thompson	animal, clinical	Y	N	N	N
FSL R8-4693	Thompson	animal, clinical	Y	N	N	N
FSL R8-6492	Thompson	animal, clinical	Y	N	N	N
FSL R8-6505	Thompson	animal, clinical	Y	N	N	N
FSL R8-6506	Thompson	animal, clinical	Y	N	N	N
FSL R8-6523	Thompson	animal, clinical	Y	N	N	N
FSL R8-6524	Thompson	animal, clinical	Y	N	N	N
FSL R8-7252	Thompson	animal, clinical	Y	N	N	N
FSL R8-7253	Thompson	animal, clinical	Y	N	N	N
FSL R8-7254	Thompson	animal, clinical	Y	N	N	N
FSL R8-7261	Thompson	animal, clinical	Y	N	N	N
FSL R8-7735	Thompson	animal, clinical	Y	N	N	N
FSL R8-7736	Thompson	animal, clinical	Y	N	N	N
FSL R8-8237	Thompson	animal, clinical	Y	N	N	N
FSL R8-8637	Thompson	animal, clinical	Y	N	N	N
FSL R8-8919	Thompson	animal, clinical	Y	N	N	N
FSL R8-8925	Thompson	animal, clinical	Y	N	N	N
FSL R8-8930	Thompson	animal, clinical	Y	N	N	N
FSL R8-9066	Thompson	animal, clinical	Y	N	N	N
FSL R8-9125	Thompson	animal, clinical	Y	N	N	N
FSL R9-0521	Thompson	animal, clinical	Y	N	N	N
FSL S10-1014	Thompson	environment, farm	Y	N	N	N
FSL S10-1031	Thompson	environment, farm	Y	N	N	N

FSL S10-1034	Thompson	environment, farm	Y	N	N	N
FSL S10-1548	Thompson	environment, farm	Y	N	N	N
FSL S10-1550	Thompson	environment, farm	Y	N	N	N
FSL S10-1552	Thompson	environment, farm	Y	N	N	N
FSL S10-1555	Thompson	environment, farm	Y	N	N	N
FSL S10-1568	Thompson	environment, farm	Y	N	N	N
FSL S10-1574	Thompson	environment, farm	Y	N	N	N
FSL A4-0730	Typhimurium	animal	Y	N	N	N
FSL A4-0731	Typhimurium	animal	Y	N	N	N
FSL A4-0732	Typhimurium	animal	Y	N	N	N
FSL A4-0733	Typhimurium	animal	Y	N	N	N
FSL A4-0734	Typhimurium	animal	Y	N	N	N
FSL A4-0765	Typhimurium	animal	Y	N	N	N
FSL A4-0766	Typhimurium	animal	Y	N	N	N
FSL A4-0767	Typhimurium	animal	Y	N	N	N
FSL P3-1552	Typhimurium	environment, farm	Y	N	N	N
FSL P3-1558	Typhimurium	environment, farm	Y	N	N	N
FSL R8-0148	Typhimurium	human, clinical	Y	N	N	N
FSL R8-0171	Typhimurium	environment, farm	Y	N	N	N
FSL R8-0172	Typhimurium	environment, farm	Y	N	N	N
FSL R8-0173	Typhimurium	environment, farm	Y	N	N	N
FSL R8-0174	Typhimurium	environment, farm	Y	N	N	N
FSL R8-0175	Typhimurium	environment, farm	Y	N	N	N
FSL R8-0281	Typhimurium	human, clinical	Y	N	N	N
FSL R8-0283	Typhimurium	human, clinical	Y	N	N	N
FSL R8-0287	Typhimurium	human, clinical	Y	N	N	N
FSL R8-0291	Typhimurium	human, clinical	Y	N	N	N
FSL R8-0812	Typhimurium	environment, farm	Y	N	N	N
FSL R8-0813	Typhimurium	environment, farm	Y	N	N	N
FSL R8-0819	Typhimurium	environment, farm	Y	N	N	N
FSL R8-0820	Typhimurium	environment, farm	Y	N	N	N
FSL R8-0920	Typhimurium	environment, farm	Y	N	N	N
FSL R8-1385	Typhimurium	environment, farm	Y	N	N	N
FSL R8-1386	Typhimurium	environment, farm	Y	N	N	N
FSL R8-1387	Typhimurium	environment, farm	Y	N	N	N
FSL R8-1952	Typhimurium	animal, clinical	Y	N	N	N
FSL R8-1953	Typhimurium	animal, non-clinical	Y	N	N	N
FSL R8-4097	Typhimurium	animal, clinical	Y	N	N	N
FSL R8-5263	Typhimurium	animal, clinical	Y	N	N	N
FSL R8-8947	Typhimurium	human, clinical	Y	N	N	N
FSL R9-1304	Typhimurium	animal, clinical	Y	N	N	N
FSL R9-1305	Typhimurium	animal, clinical	Y	N	N	N
FSL R9-1307	Typhimurium	animal, clinical	Y	N	N	N
FSL R9-1308	Typhimurium	animal, clinical	Y	N	N	N
FSL R9-1310	Typhimurium	animal, clinical	Y	N	N	N
FSL S10-1134	Typhimurium	environment, farm	Y	N	N	N
FSL S10-1269	Typhimurium	environment, farm	Y	N	N	N
FSL S10-1766	Typhimurium	environment, farm	Y	N	N	N
FSL S5-0397	Typhimurium	human	Y	N	N	N
FSL S5-0452	Typhimurium	human	Y	N	N	N
FSL S5-0462	Typhimurium	human	Y	N	N	N
FSL S5-0473	Typhimurium	human	Y	N	N	N
FSL S5-0476	Typhimurium	human	Y	N	N	N
FSL R8-8956	Typhimurium var. O:5-	human, clinical	Y	N	N	N
FSL R8-9657	Typhimurium var. O:5-	human, clinical	Y	N	N	N
FSL R8-9661	Typhimurium var. O:5-	human, clinical	Y	N	N	N
FSL R8-9669	Typhimurium var. O:5-	human, clinical	Y	N	N	N
FSL R8-4925	Montevideo	human, clinical	Y	Y	Y	Y
FSL R8-4926	Montevideo	human, clinical	Y	Y	Y	Y
FSL S5-0386	Thompson	animal	Y	N	N	N
FSL R8-4829	Oranienburg	animal, clinical	Y	Y	Y	Y
FSL S5-0415	Enteritidis	human, clinical	Y	N	N	N

FSL S5-0536	Typhimurium	human, clinical	Y	N	N	N
FSL S5-0639	Newport	human, clinical	Y	N	N	N

a Three isolates (FSL A4-0591, FSL R6-0620, FSL S5-0510) had an amplicon for the PCR screen for *cdtB*, but did not yield a PCR product for the full gene. In addition, all three of these isolates did not yield an amplicon with *pltA* and *pltB* primers. Hence these isolates were classified as S-CDT negative.

HeLa cells were grown in Eagle's Minimum Essential Medium (EMEM; Gibco-Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco-Invitrogen), and were incubated at 37°C with 5% CO_2. For infection, HeLa cells were grown in 6-well or 12-well plates (Corning, Corning, NY) to desired confluency (40-50% confluency for flow cytometry analyses, or 40-70% for immunofluorescence analyses).

PCR screen for S-CDT genes *pltA, pltB,* and *cdtB*. Isolates were plated on BHI agar plates from frozen glycerol stocks, followed by overnight incubation at 37°C. Cell lysates were prepared by inoculating 100 µl of sterile water with bacterial cells, followed by heating at 95°C for 15 min in a thermocycler. Each isolate was screened for genes encoding the 3 components of S-CDT (*pltA, pltB,* and *cdtB*) using the primers and their respective annealing temperatures listed in Table 3.3. Screening was performed using 2 PCRs. The first PCR contained primers to amplify (i) a portion of the *16S* rDNA (included as positive control), and (ii) an internal fragment of *pltA.* The second PCR was used to simultaneously amplify internal regions of *pltB* and *cdtB.* Thermocycling conditions included an initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at either 48°C or 50°C (Table 3.3) for 30 s, and extension at 72°C for 1 min; a final extension at 72°C for 7 min was also included. PCR products were visualized using agarose gel electrophoresis. In the event that PCR amplification did not yield amplicons for *16S* rDNA, a new lysate was prepared and both PCRs were re-performed.

Sequencing of full-length *pltA, pltB,* and *cdtB* for phylogenetic comparison. Full-length gene segments were amplified for 5 representative isolates (with the exception of serotype Mississippi, which only had 4 isolates that were S-CDT-positive) from each serotype or clade

that was identified as S-CDT-positive. Primers were designed to anneal to regions upstream and downstream of *pltA*, *pltB,* and *cdtB* (see Table 3.3).

TABLE 3.3. Primers used in this study

Primer Name	Primer Sequence (5' to 3')	Annealing Temperature[a]	Expected Amplicon Size (bp)
RM45cdtBintF_2	CTGCGCTAATATCAGTGACTAC	48°C	639
RM46cdtBtintR_2	CAACCCTTTGTGAATAAGGTGC		
RM47pltBintF_2	ACGATGATAGTCCCACAAGAGC	48°C	243
RM48pltBintR_2	CCTACTACTCAGACGAAGTTATC		
RM49pltAintF_2	ATCACGCCAGGATTAGACGTAG	50°C	313
RM50pltAintR_2	GCTATATTGCAACAACCTCAAGTG		
16s-PEU7	GCAAACAGGATTAGATACCC	50°C	700
16s-P3SH	CTACGGTTACCTTGTTACGACTT		
RM23PltAupstream	CCAGACCACTAATAAACGGTCTG	60-50°C	801
RM24PltAdownstream	TATCATCGTGCAGTGCGA		
RM25PltBupstream	GACTGGACTGTGATGTGGTG	60-50°C	545
RM26PltBdownstream	GTTGAGTCCACACGATACAC		
RM97cdtBupF	CAACGTCATGAAACAATGGGTTATG	57°C	935
RM96cdtBdownR	ATATTCTGCACCTTACGCTCAAAGTAC		

[a]Annealing temperature used in PCR amplification; annealing temperatures reported as a range were performed using a range of temperatures as a part of a touchdown PCR

PCR amplification was performed using a touchdown method (for *pltA* and *pltB*) with initial denaturation at 94°C for 3 min, followed by 20 cycles of denaturation at 94°C for 30 s with annealing temperature decreasing by 0.5°C per cycle (from 60°C at cycle 1 to 50°C at cycle 20; Table 3.3) and extension at 72°C for 1.5 min; an additional 20 cycles of amplification were then performed using an annealing temperature of 50°C with a final extension at 72°C for 7 min. For *cdtB*, amplification was performed using the following thermocycling conditions; 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and elongation for 1 min at 72°C, followed by a final extension at 72°C for 5 min. PCR products were treated with 5 units exonuclease I (Affymetrix, Santa Clara, CA) and 5 units shrimp alkaline phosphatase (Affymetrix), followed by Sanger sequencing performed by the Cornell Biotechnical Resource Center (Cornell University, Ithaca, NY). Sequences were edited using Sequencher software (Gene Codes Corporation; Ann Arbor, MI). Phylogenetic analyses were performed using Mega Software (Mega Version 6 Software). GenBank accession numbers for all S-CDT subunits sequenced are available in Table 3.4.

TABLE 3.4. Description of GenBank accession numbers for *pltA*, *pltB*, and *cdtB* sequences for *Salmonella* strains characterized in this study

Sequence Description	GenBank Accession ID
FSL A4-0564_pltB	KX810866
FSL A4-0642_pltB	KX810867
FSL A4-0594_pltB	KX810868
FSL A4-0644_pltB	KX810869
FSL A4-0668_pltB	KX810870
FSL M8-0480_pltB	KX810871
FSL M8-0489_pltB	KX810872
FSL M8-0491_pltB	KX810873
FSL M8-0494_pltB	KX810874
FSL M8-0495_pltB	KX810875
FSL R8-1549_pltB	KX810876
FSL R8-2004_pltB	KX810877
FSL R8-2005_pltB	KX810878
FSL R8-2106_pltB	KX810879
FSL R8-2124_pltB	KX810880
FSL R8-2165_pltB	KX810881
FSL R8-2455_pltB	KX810882
FSL R8-2779_pltB	KX810883
FSL R8-2533_pltB	KX810884
FSL R8-7996_pltB	KX810885
FSL S5-0642_pltB	KX810886
FSL A4-0564_pltA	KX810887
FSL A4-0594_pltA	KX810888
FSL A4-0642_pltA	KX810889
FSL A4-0644_pltA	KX810890
FSL A4-0668_pltA	KX810891
FSL M8-0480_pltA	KX810892
FSL M8-0489_pltA	KX810893
FSL M8-0491_pltA	KX810894
FSL M8-0494_pltA	KX810895
FSL M8-0495_pltA	KX810896
FSL R8-2004_pltA	KX810897
FSL R8-2005_pltA	KX810898
FSL R8-2106_pltA	KX810899
FSL R8-2124_pltA	KX810900
FSL R8-2165_pltA	KX810901
FSL R8-2455_pltA	KX810902
FSL R8-2533_pltA	KX810903
FSL R8-1549_pltA	KX810904
FSL R8-2779_pltA	KX810905

FSL R8-7996_pltA	KX810906
FSL A4-0564_cdtB	KX810907
FSL A4-0594_cdtB	KX810908
FSL A4-0642_cdtB	KX810909
FSL A4-0644_cdtB	KX810910
FSL A4-0668_cdtB	KX810911
FSL M8-0480_cdtB	KX810912
FSL M8-0489_cdtB	KX810913
FSL M8-0491_cdtB	KX810914
FSL M8-0494_cdtB	KX810915
FSL M8-0495_cdtB	KX810916
FSL R8-1549_cdtB	KX810917
FSL R8-2004_cdtB	KX810918
FSL R8-2005_cdtB	KX810919
FSL R8-2106_cdtB	KX810920
FSL R8-2124_cdtB	KX810921
FSL R8-2165_cdtB	KX810922
FSL R8-2455_cdtB	KX810923

7-Gene MLST. For selected isolates, multi locus sequence typing (MLST) was performed as described previously, using internal fragments of housekeeping genes a*roC, dnaN, hemD, hisD, purE, sucA,* and *thrA* (30). Sequences were edited and compared to the *S. enterica* MLST database, which allows for assignment of MLST sequence types (30).

***Salmonella* infection of HeLa cells.** Bacterial cultures were plated from frozen glycerol stocks onto BHI agar plates, followed by incubation at 37°C for 22-24 h. Subsequently, single colonies were used to inoculate 5 ml aliquots of LB (pH 8; 0.3 M NaCl) broth, followed by incubation under static conditions at 37°C for 14 $\pm$ 1 h. These cultures were then sub-cultured 1:100 into fresh 5 ml aliquots of LB (pH 8; 0.3 M NaCl) broth, followed by incubation at 37°C under static conditions until bacteria reached early to mid-log phase (OD_{600} of 0.4 $\pm$ 0.1). Treatments (i.e. level of inoculum and strain of NTS used) were randomly assigned to HeLa cells seeded in 6-well or 12-well plates. HeLa cells were infected with bacterial cultures at multiplicities of infection (MOI) of approximately 5 and 10. After incubation of the infected cells for approximately 1 h at 37°C (with 5% CO_2), HeLa cells were washed 3 times with phosphate

buffered saline (PBS), followed by incubation with EMEM supplemented with 100 μg/ml gentamicin (Gibco) for 1.25 h at 37°C to kill extracellular bacteria. Subsequently, HeLa cells were washed an additional 3 times with PBS, and were then maintained in EMEM containing 10 μg/ml gentamicin (Gibco) to prevent recurrent infection and bacterial outgrowth during incubation.

Flow Cytometry. After the selected incubation periods, HeLa cells were washed once with PBS, and were harvested using 0.5% Trypsin EDTA (Gibco). Cells were fixed in 70% ethanol at -20°C for at least 3 h. Ethanol-fixed cells were permeabilized with PBS containing 0.1% Tween-20 (PBS-T) and bovine serum albumin (1 g/100 ml) (Sigma-Aldrich, St. Louis, MO) at room temperature for 10 min. Cells were subsequently stained (10 min at room temperature) with a solution containing propidium iodide (PI; ThermoFisher Scientific, Waltham, MA) at a final concentration of 50 μg/ml and RNase A (Sigma-Aldrich) at a final concentration of 100 μg/ml. Stained cells were held at 4°C for no longer than 4 h, prior to DNA content analysis using the BD FACSARIA. Cells were gated to exclude doublets and multiplets, as described previously (31).

Immunofluorescence Staining. HeLa cells grown on 12 mm coverslips (ThermoFisher Scientific) seeded in 12-well plates were infected with NTS strains at a MOI of approximately 5, as described above. At 48 hours post infection (hpi), cells were washed with PBS and then fixed with 4% paraformaldehyde in PBS at room temperature for 15-20 min. Slides were washed with PBS, followed by permeabilization with 0.5-1% Triton-X 100 in PBS at room temperature for 10 min. Permeabilized cells were then blocked with 10% horse serum (Gibco) in PBS-T for 1 h at room temperature. Incubation with primary antibodies was performed for 1 h at room temperature using the following dilution factors in PBS-T: polyclonal goat anti-Salmonella antibody (KPL antibodies, Gaithersburg, MA; 1:500), mouse anti-γ-H2AX (EMD Millipore, Billerica, MA; 1:500), rabbit anti-53BP1 (Novus Biologicals, Littleton, CO; 1:500). Incubation with secondary antibodies diluted 1:200, except where noted, in PBS-T was performed for 1 h at room temperature; antibodies used were donkey anti-mouse conjugated to Alexa 405 (Abcam,

Cambridge, MA); donkey anti-goat conjugated to Alexa 488; donkey anti-rabbit conjugated to Alexa 555 (diluted 1:500); and donkey anti-mouse conjugated to Alexa 647 (all ThermoFisher Scientific). Nuclei were stained with either 4',6-diamidino-2-phenylindole (DAPI) at a final concentration of 1 μg/ml, or PI at a final concentration of 40 μg/ml for 5 min at room temperature. Slides were subsequently mounted onto microscope slides with glycergel (Dako, Carpinteria, CA) and were imaged using a Zeiss 710 confocal microscope. Two Z-stacks (representing 2 independent fields of view) were collected and analyzed per slide. Images were processed with FIJI software, and cells were counted using the cell counter plug-in (32). At least 90 nuclei were analyzed per slide to identify cells that had more than four 53BP1 positive foci and were also positive for γH2AX; these cells were designated as 53BP1 and γH2AX positive cells (or "double positive cells"). The observer was blinded to the treatment while collecting and analyzing images.

Cell-free supernatant intoxication. Media supernatant was collected at 48 hpi from HeLa cells infected with different *Salmonella* strains, and was filtered through a 0.2 μm filter to remove bacterial cells. The resulting cell-free supernatant was co-incubated with fresh HeLa cell monolayers for 24 h. Following incubation, cells were fixed for flow cytometry, or for immunofluorescence microscopy, as described above. Cell-free supernatants were also tested after heat treatment (70°C or 95°C for 10 min).

Statistical Analysis. All statistical analyses were performed using R software (version 3.0.2; R-project, Vienna, Austria). Linear mixed effects models were used to assess significant associations between S-CDT status (presence or absence of S-CDT-encoding genes, or WT vs. Δ*cdtB* isogenic mutants) and either (i) proportions of cell populations in G2/M, or (ii) proportions of cells with an activated DDR as determined using immunofluorescence staining for 53BP1 and γH2AX foci. MOI and S-CDT status were included as fixed effects. To account for potential variability due to (i) the passage number of the HeLa cells, and (ii) the *Salmonella* strain, these variables were included in models as random effects. An interaction term was included for S-CDT status and the MOI, when appropriate. Data were transformed by taking the

log_{10}, natural log, square root, or performing a rank transformation, when appropriate to obtain a normal distribution of the residuals. Data shown in Figs represent untransformed data. The lsmeans (least square means) package was used to perform pairwise comparisons among treatments, and P-values were adjusted using the Tukey method to account for multiple comparisons. The 90% confidence interval for the binomial proportion of human Mississippi isolates positive for S-CDT was estimated using the Wilson score interval.

Data Availability. All sequence data is available in NCBI GenBank (see Table 3.4 for accession numbers), and in the publicly available online database www.foodmicrobetracker.com.

RESULTS

S-CDT is conserved among isolates of NTS serotypes Javiana, Montevideo, and Oranienburg, while for serotype Mississippi isolates genes encoding S-CDT are clade-associated. A PCR screen used to detect internal gene fragments for *pltA*, *pltB*, and *cdtB* showed that, among the 21 NTS serotypes, all *S. enterica* serotypes Javiana, Montevideo, and Oranienburg isolates screened (n = 50 for each serotype) encoded all 3 toxin components. For serotype Mississippi, only 4 of the 8 isolates tested were S-CDT-positive (see Table 3.5).

TABLE 3.5 Distribution of S-CDT-encoding genes among 21 nontyphoidal *Salmonella enterica* serotypes most commonly isolated from human cases in the US in 2011

Serotype	Serogroup	Number of reported laboratory confirmed human clinical cases in 2011[a]	Proportion of S-CDT-positive isolates[b]	
Enteritidis	D_1	7,553	0%	(0/50)
Typhimurium[c]	B	6,131	0%	(0/50)
Newport	C_2-C_3	5,211	0%	(0/50)
Javiana	D_1	2,937	100%	(50/50)
I 4,[5],12:i:-	B	1,339	0%	(0/50)
Montevideo	C_1	1,196	100%	(50/50)
Heidelberg	B	1,103	0%	(0/50)
Muenchen	C_2-C_3	984	0%	(0/48)
Infantis	C_1	910	0%	(0/50)
Braenderup	C_1	739	0%	(0/50)
Oranienburg	C_1	721	100%	(50/50)
Saintpaul	B	709	0%	(0/50)
Mississippi	G	549	50%	(4/8)
Thompson	C_1	536	0%	(0/50)
Agona	B	505	0%	(0/50)
Bareilly	C_1	429	0%	(0/22)
Berta	D_1	321	0%	(0/20)
Anatum	E_1	282	0%	(0/50)
Hartford	C_1	241	0%	(0/18)
I 13,23:b:-	G	218	0%	(0/1)
Hadar	C_2-C_3	205	0%	(0/47)

[a] Reported laboratory confirmed human clinical cases in 2011 according to CDC National Salmonella Surveillance report 2011
[b]Presence of all S-CDT components (*pltA, pltB,* and *cdtB*) as determined by PCR amplification; proportion of S-CDT-positive strains tested per serotype
[c]Serotype Typhimurium includes Typhimurium variant O:5-

Subsequent 7-gene MLST characterization of the 8 serotype Mississippi isolates indicated that this serotype is polyphyletic and that these isolates represented 2 distinct clades (Fig. 2.1). S-CDT-encoding genes were present in all 4 isolates with MLST sequence type (ST) 425 (see Fig. 2.1) (30), while the 4 isolates in the other clade (representing ST 448 and a new ST) were negative for the S-CDT genes. Two other *Salmonella* Mississippi STs (i.e. ST 356 and 764), obtained from the MLST database, also clustered into the S-CDT-negative clade (30). Interestingly, the S-CDT-negative Mississippi clade clustered with *S.* Typhi (S-CDT-positive), suggesting that this clade may have lost the S-CDT genes (Fig. 3.1).

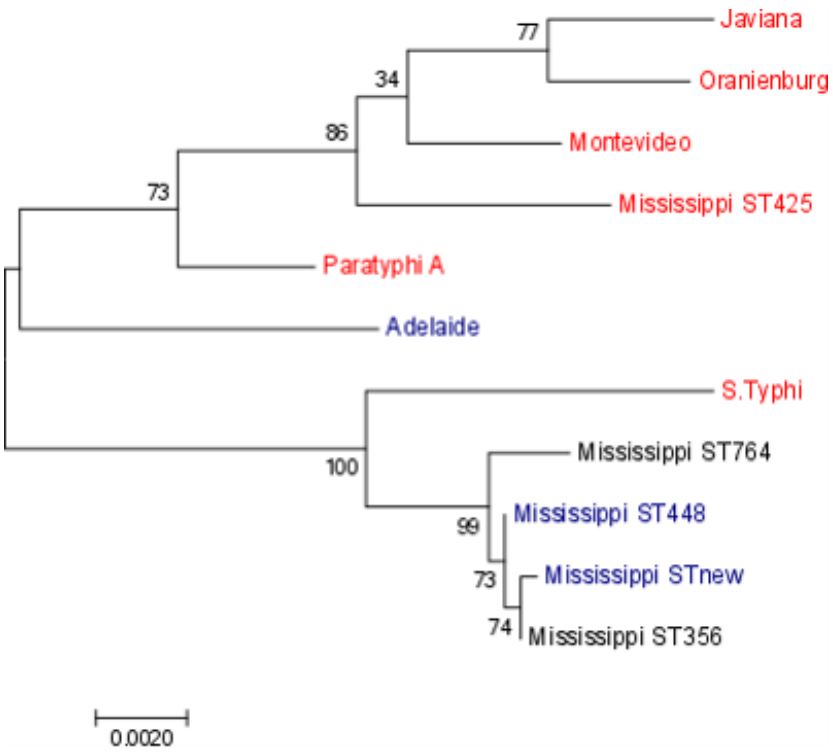

FIGURE 3.1 Genes encoding S-CDT are clade restricted for serotype Mississippi. Maximum-likelihood tree showing phylogenetic clades based on concatenated 7-gene MLST sequences for serotype Mississippi, closely related serotypes Adelaide, Paratyphi A, and Typhi (22), and S-CDT positive serotypes Javiana, Oranienburg, and Montevideo, which are included for comparison. MLST data for the Mississippi isolates designated as STnew, ST356, ST425, ST448, and ST764 were obtained here; all other sequence data were extracted from the MLST database (30). Branch lengths represent a substitution rate of 2 nucleotides per 1000. Numbers at nodes represent bootstrap values based on 1000 repetitions. Blue represents S-CDT-negative MLST sequence types and serotypes as determined in this study, and by den Bakker et al. (22); red represents S-CDT-positive MLST sequence types and serotypes. S-CDT status for serotype Mississippi ST764 and ST356 are unknown, and these STs are thus shown in black lettering.

Based on CDC data on the frequency of different NTS serotypes among human clinical cases in the US, the 3 serotypes Javiana, Montevideo, and Oranienburg (where all isolates were S-CDT-positive) represent 4,854 of the 32,819 reported cases of nontyphoidal salmonellosis caused by the 21 NTS serotypes screened (listed in Table 3.5). Serotype Mississippi on the other hand accounted for 549 reported cases; with 4/8 Mississippi isolates testing as S-CDT positive, we can estimate that the binomial proportion of human Mississippi isolates positive for S-CDT is 0.5 with a 90% confidence interval of 0.25 to 0.75. Based on this, infections with S-CDT-

encoding Mississippi strains account for approximately 275 cases per year (90% C.I.: 137 - 412). Overall, S-CDT-positive isolates among these 21 human clinical NTS associated serotypes were estimated to cause approximately 5,129 reported human cases in the US. As Scallan *et al*. (17) previously suggested that only 1 out of each 29.3 cases of nontyphoidal salmonellosis is reported (90% CI 21.8 - 38.4), we estimated that approximately 150,280 cases (90% C.I.: 111,812 – 196,954) of salmonellosis in the US are caused by S-CDT-positive isolates representing serotypes Javiana, Montevideo, Oranienburg, and Mississippi.

***pltA, pltB,* and *cdtB* are highly conserved among NTS serotypes encoding S-CDT.** To characterize the sequence conservation of *pltA, pltB,* and *cdtB* (encoding S-CDT subunits PltA, PltB, and CdtB, respectively) we sequenced PCR amplicons for each of these genes for 5 representative isolates from each of the S-CDT-positive serotypes, except for serotype Mississippi where all 4 S-CDT-positive isolates were characterized. Overall, the predicted peptide products of *pltA, pltB,* and *cdtB* were highly conserved across serotypes (including *S.* Typhi strain CT-18) with 98.3%, 94.9%, and 99.3% of amino acids (aa) conserved, respectively. DNA sequences for *pltA, pltB,* and *cdtB* were also highly conserved, with 98.1%, 96.1%, and 99.4% of nt identical among all isolates, respectively.

The CdtB subunit was the most conserved among the toxin-encoding subunits. Among the isolates analyzed, we found 2 CdtB sequence types. All serotype Javiana, Montevideo, and Oranienburg isolates had the same CdtB aa sequences (see Table 3.6), which were identical to Typhi strain CT-18 (designated CdtB sequence type 1). CdtB sequence type 2 included all S-CDT-positive serotype Mississippi isolates, which had aa substitutions at residues 217 (Thr vs. Ala in CT-18) and 223 (Ala vs. Glu in CT-18). The cysteine residue (Cys269) essential for anchoring CdtB and PltA (12) was conserved among the predicted CdtB peptide sequences for all S-CDT-positive NTS isolates analyzed.

TABLE 3.6 Amino acid differences in S-CDT subunits between Typhi and nontyphoidal serotypes.

Subunit	AA Residue[a]	AA (nt codon) in CT-18[b]	AA (nt codon) in Serotype[c]	Serotypes with Substitution
CdtB	217	Ser (**G**CC)	Thr (**A**CC)	Mississippi
	223	Glu (G**A**A)	Ala (G**C**A)	Mississippi
PltB[d]	3	Met (AT**G)**	Ile (AT**A)**	Javiana, Montevideo, Mississippi, Oranienburg
	4	Ser (A**G**T)	Asn (A**A**T)	Javiana, Montevideo, Mississippi, Oranienburg
	6	Tyr (T**A**T)	Phe (T**T**T)	Javiana, Montevideo, Mississippi, Oranienburg
	29	Asn (AA**T**)	Lys (AA**A**)	Javiana, Montevideo, Oranienburg
	50	Ser (**A**GT)	Gly (**G**GT)	Javiana, Oranienburg
	65	Thr (A**C**A)	Ile (A**T**A)	Javiana, 1 of the 5 Oranienburg isolates
	116	Thr (**A**CC)	Ala (**G**CC)	4 of the 5 Oranienburg isolates
PltA[e]	6	Phe (**T**TC)	Leu (**C**TC)	Javiana, Oranienburg
	169	Ala (**G**CC)	Ser (**T**CC)	Javiana, Montevideo, Oranienburg
	215	His (CA**T**)	Gln (CA**A**)	Javiana, Montevideo, Mississippi, Oranienburg
	227	Met (**A**TG)	Val (**G**TG)	Javiana

[a]AA = amino acid residue in subunit
[b]three letter abbreviation for amino acid residue in *S. enterica* serotype Typhi strain CT-18; codon encoding the amino acid residue is shown in parenthesis. The specific SNP causing the amino acid change is shown in bold font.
[c]three letter abbreviation for amino acid residue in nontyphoidal serotypes listed in right-most column; codon encoding the amino acid residue is shown in parenthesis. The specific SNP causing the amino acid change is shown in bold font.
[d]*Salmonella* Oranienburg strain FSL A4-0642 showed the same amino acid substitution in PltB as the 5 Javiana isolates tested
[e]In addition to the aa differences listed here, isolate FSL R8-1549 (serotype Mississippi) had a SNP resulting in an amino acid change from glycine to serine at position 211; this genotype was not detected in any other isolates

The key aa residues for PltA, the ribosyl transferase subunit, were also conserved. Both the PltA aa residue implicated in binding to the CdtB subunit (Cys214), as well as the catalytic residue (Glu133) were conserved among all isolates. A total of 4 amino acid substitutions were identified, compared to Typhi CT-18 (see Table 3.6). Serotype-specific amino acid substitutions included (i) a His215 residue in Typhi strain CT-18 (compared to Gln215 for all other serotypes), and (ii) a Val227 residue in all Javiana isolates (compared to a Met227 for all other isolates) (see Table 3.6).

The PltB subunit was the most variable of the toxin subunits, with 7 variable aa residues (see Table 3.6). While for 3 variable sites (residues 3, 4, and 29), the PltB sequence for S. Typhi CT-18 differed from all other isolates, other residues showed different aa substitution patterns. For example, all serotype Javiana and 1 serotype Oranienburg isolate showed an Ile65 (in place

of Thr65 in all serotype Montevideo isolates, the 4 remaining serotype Oranienburg isolates, and strain Typhi CT-18). For 1 Oranienburg isolate, the complete PltB aa sequence was identical to the Javiana isolates. We confirmed that this was not due to misidentification of this isolate as the 7-gene MLST sequence for this isolate matched STs of other serotype Oranienburg isolates. All key residues involved in binding to sugar moieties (Tyr33, Ser35, and Lys59) were conserved across all serotypes (12).

Infection with S-CDT-positive serotypes results in G2/M cell cycle arrest, while infection with S-CDT-negative serotypes does not. To determine if the presence of S-CDT-encoding genes altered the outcome of infection at the cellular level we infected HeLa cells with *S. enterica* isolates representing S-CDT-positive and S-CDT-negative serotypes. Overall, we found that HeLa cell populations infected with isolates representing S-CDT-positive serotypes (i.e. Javiana, Montevideo, Mississippi, and Oranienburg) showed a significantly higher proportion of cells in the G2/M phase (Fig. 3.2A and 3.2B), compared to HeLa cells infected with S-CDT-negative serotypes (i.e., Enteritidis, Newport, and Typhimurium), both at MOI 5 ($p = 0.016$) and at MOI 10 ($p = 0.011$). For example, for an MOI of 10, the proportion of HeLa cells in the G2/M phase ranged from 25.9% to 89.7% for the different S-CDT-positive isolates, and from 13.8% to 21.1% for the S-CDT-negative isolates (see Fig. 3.2A). For HeLa cells infected with S-CDT-negative serotypes, the proportion of cells in the G2/M phase, did not significantly differ from the proportion of uninfected cells in the G2/M phase, regardless of MOI (MOI 5 $p = 0.999$; MOI 10 $p = 0.998$). We also found a significant effect ($p < 0.0001$) of the isolate used for infection, on the proportion of HeLa cells in G2/M phase. Post-hoc Tukey's specifically showed significant differences in the magnitude of G2/M arrest caused by the different S-CDT-positive isolates tested (Fig. 3.2); HeLa cells infected with either serotype Mississippi or Oranienburg both showed a significantly lower ($p < 0.05$) proportion of HeLa cells in the G2/M phase, as compared to HeLa cells infected with either Javiana or Montevideo. Importantly, even for infections with Mississippi and Oranienburg isolates, the proportion of HeLa cells in the G2/M phase was still significantly ($p < 0.05$) higher as compared to any of the infections involving S-

CDT-negative isolates. Overall, our results indicate that infection with S-CDT-encoding serotypes is associated with a significant increase in the proportion of cell populations arrested in the G2/M phase, indicative of DNA damage.

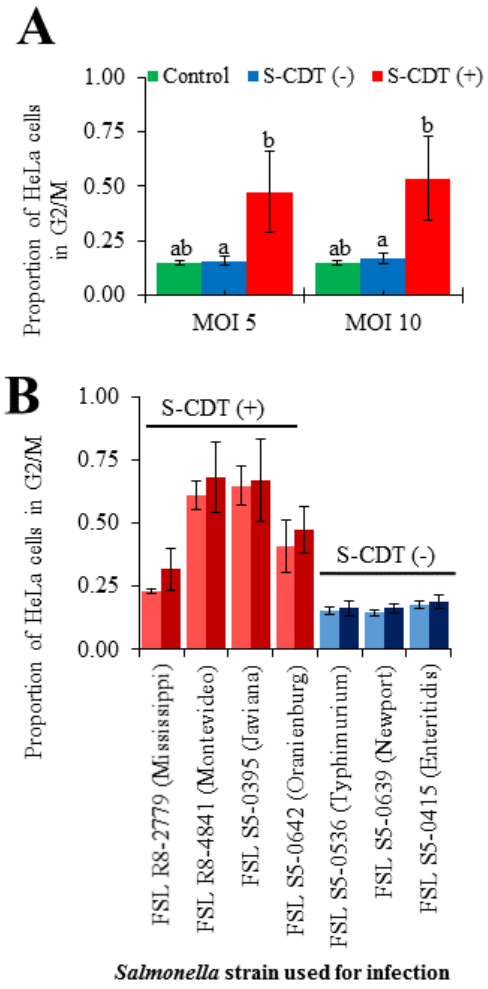

FIGURE 3.2 Infection with NTS serotypes encoding S-CDT results in a significantly higher proportion of HeLa cells arrested in G2/M. HeLa cells were infected with isolates representing S-CDT-positive serotypes (Mississippi, Montevideo, Javiana, and Oranienburg) and S-CDT-negative serotypes (Enteritidis, Typhimurium, and Newport), at multiplicities of infection (MOI) of approximately 5 and 10. At 48 hpi, cells were fixed and stained with PI. DNA content was analyzed by flow cytometry, and cells were gated to exclude multiplets. Least square means from a linear mixed effects model were used to determine significant differences in proportions of HeLa cells in the G2/M phase for cells infected with the 2 MOIs. Results represent 4 independent experiments. (A) Bar chart showing average proportions of cell populations in G2/M phase among HeLa cells infected with S-CDT-negative (blue bars) or S-CDT-positive serotypes (red bars) at 2 MOIs (5 and 10, shown on X-axis); data shown are the averages for the 4 S-CDT-positive and the 3 S-CDT-negative strains. Green bars represent values for uninfected control cells (4 independent replicates). Bars that do not share identical letters represent statistically significantly different proportions of cells in G2/M phase ($p < 0.05$). (B) Bar chart showing G2/M arrest resulting from infection with the individual *Salmonella* strains used in this study (see Table 1). Light blue and red bars represent infection at MOI of 5, and dark blue and dark red bars represent infection at MOI of 10. Statistical models are based on log-transformed data; non-transformed data are shown. Error bars represent standard deviations.

S-CDT-encoding serotypes activate the DNA damage response while S-CDT-negative, or S-CDT-null serotypes do not. CdtB, the active component of S-CDT has nuclease activity *in vitro*, and activates the host cell's DDR (3, 12, 16). To determine if infection with NTS serotypes encoding S-CDT resulted in an activated DDR among infected HeLa cell populations, we used immunofluorescence staining to detect 53BP1 foci, and then confirmed this by also staining for phosphorylated Serine 139 on H2AX (yielding γH2AX) (33, 34). HeLa cell populations infected with S-CDT-encoding *Salmonella* had a significantly higher proportion of cell populations with both 53BP1 and γH2AX foci ("double positive cells"; Fig. 3.3A and 3.3B), compared to either HeLa cells infected with S-CDT-negative isolates ($p < 0.001$) or uninfected controls ($p = 0.003$).

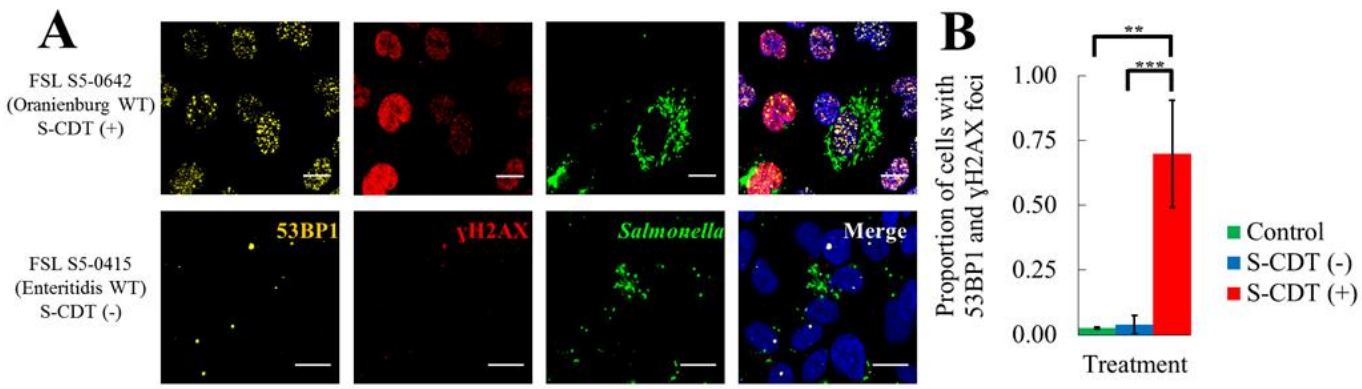

FIGURE 3.3 Infection with S-CDT-encoding serotypes is associated with activation of the DNA damage response. HeLa cells grown on glass coverslips were infected with *Salmonella* strains at a multiplicity of infection of approximately 5. Cells were fixed at 48 hpi and were stained with antibodies recognizing 53BP1, γH2AX, and *Salmonella* cells. DAPI was included as a nucleic acid stain. (A) Representative images for strain FSL S5-0642 (serotype Oranienburg; S-CDT-positive) and FSL S5-0415 (serotype Enteritidis; S-CDT-negative); data for other strains are shown in Fig. S1. Scale bars represent 25 μm. (B) A mixed effects linear model was used to determine whether the proportion of HeLa cells that were positive for 53BP1 and γH2AX foci at 48 hpi, differed between S-CDT-positive and negative serotypes and uninfected controls. Data represent 2 technical replicates from 2 independent experiments for each serotype. The S-CDT-negative bar represents the average of all data for HeLa cells infected with serotypes Typhimurium, Newport, and Enteritidis; the S-CDT-positive bar represents the average for all data for HeLa cells infected with serotypes Javiana, Montevideo, Mississippi, and Oranienburg. *** represents $p < 0.001$; ** represents $p < 0.01$. While the data were transformed for statistical analyses, non-transformed data are shown. Bars represent standard deviations.

This effect was consistent for all isolates tested (Fig 3.4). Specifically, 27.1% to 91.6% of HeLa cells infected with S-CDT-positive isolates representing serotypes Javiana, Montevideo, Mississippi, and Oranienburg showed both 53BP1 and γH2AX foci ("double positive cells"), compared to 0.0% to 9.3% of HeLa cells infected with isolates representing S-CDT-negative serotypes. For HeLa cells infected with isolates representing S-CDT-negative serotypes, the proportion of double-positive cells (i.e. cells with both 53BP1 and γH2AX foci) also did not differ from uninfected controls ($p = 0.992$). As 53BP1 and γH2AX foci have been shown previously to localize to sites of DNA damage, these results also support the hypothesis that HeLa cells infected with S-CDT-positive serotypes sustain more DNA damage than HeLa cells infected with S-CDT-negative serotypes (35). Therefore, these results suggest that infection with isolates representing different S-CDT-encoding NTS serotypes results in DNA damage which activates the host cell DDR, while infection with isolates representing S-CDT-negative serotypes does not.

To confirm that the observed activation of the host cell DDR was due to CdtB, we infected HeLa cells with Δ*cdtB* isogenic mutants of serotype Javiana and Montevideo isolates. Deletion of *cdtB* abolished the ability of these strains to activate the host cell DDR, as the proportion of Δ*cdtB* infected HeLa cells that were double positive for 53BP1 and γH2AX foci was not significantly different from uninfected controls ($p = 0.805$). HeLa cell populations infected with WT isolates of Javiana and Montevideo also had significantly ($p = 0.004$) higher proportions of cells with an activated DDR (i.e., with 53BP1 and γH2AX foci) as compared to HeLa cell populations infected with the Δ*cdtB* isogenic mutants (Fig. 3.5A and 3.5B); immunofluorescence images indicated similar levels of intracellular *Salmonella* in HeLa cells infected with WT parent strains and their respective Δ*cdtB* isogenic mutants, suggesting that *cdtB* did not influence rates of invasion or intracellular survival within HeLa cells. Taken together, these results suggest that CdtB is essential for the activation of the DDR response among cells infected with S-CDT-positive NTS serotypes.

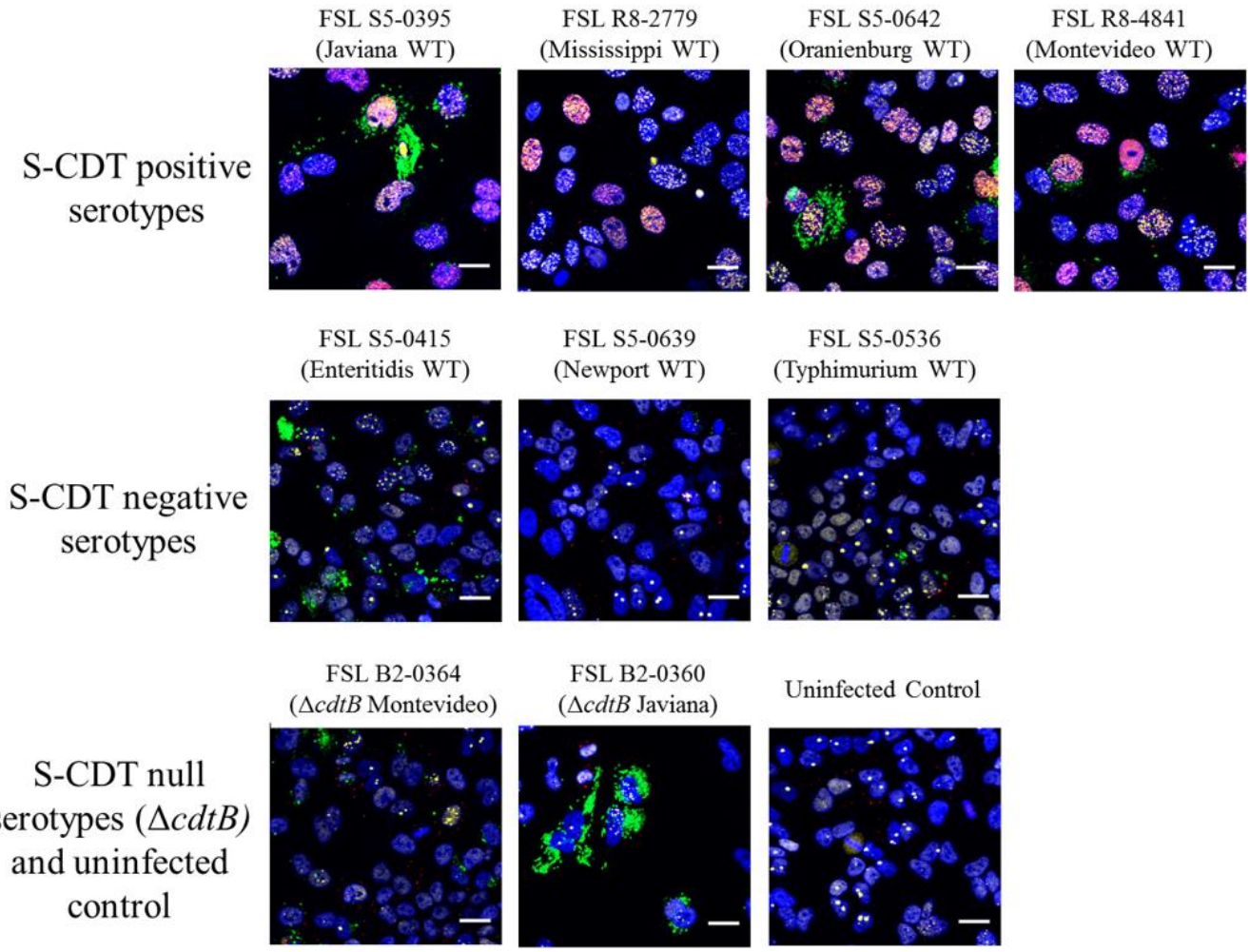

FIGURE 3.4 Infection with S-CDT-positive serotypes activates the host DNA damage response while infection with S-CDT-negative serotypes does not. HeLa cells were infected with S-CDT-positive, S-CDT-negative, and Δ*cdtB* isogenic mutants, at a multiplicity of infection of approximately 5. Immunofluorescence staining was performed to detect 53BP1 and γH2AX foci at 48 hpi. Representative images are included for all serotypes. Scale bars represent 25 μm.

***cdtB* is required for the G2/M cell cycle arrest associated with infection with S-CDT-positive serotypes.** To confirm that the G2/M arrest was due to CdtB we compared the cell cycle progression of populations of HeLa cells infected with the WT Javiana and Montevideo strains and their Δ*cdtB* isogenic mutants. HeLa cells infected with the WT Javiana and Montevideo strains had a significantly higher proportion of cells in the G2/M phase at both MOI 5 ($p < 0.001$) and MOI 10 ($p < 0.001$), compared to cell populations infected with Δ*cdtB* isogenic mutants (Fig. 3.5C). There was no statistically significant effect of MOI (5 or 10) on the proportion of HeLa cells that arrested in G2/M after infection with S-CDT-positive serotypes ($p = 0.831$), indicating that MOI did not have a significant effect on the observed G2/M cell cycle arrest, even though immunofluorescence imaging showed that only a small proportion of HeLa cells were infected with *Salmonella* (see Fig 3.5; at an MOI of approximately 5, only a median of 10% of HeLa cells were infected). Importantly, for either an MOI of 5 ($p = 0.167$) or an MOI of 10 ($p = 0.149$), the proportion of HeLa cells in the G2/M phase, following infection with Δ*cdtB* *Salmonella* isolates, was not significantly different from the proportion of uninfected controls in the G2/M phase. Deletion of *cdtB* thus abolished the ability of the S-CDT-positive serotypes Javiana and Montevideo to arrest HeLa cells in the G2/M phase (Fig. 3.5C), demonstrating that CdtB is required for the G2/M cell cycle arrest associated with infection involving S-CDT-positive serotypes.

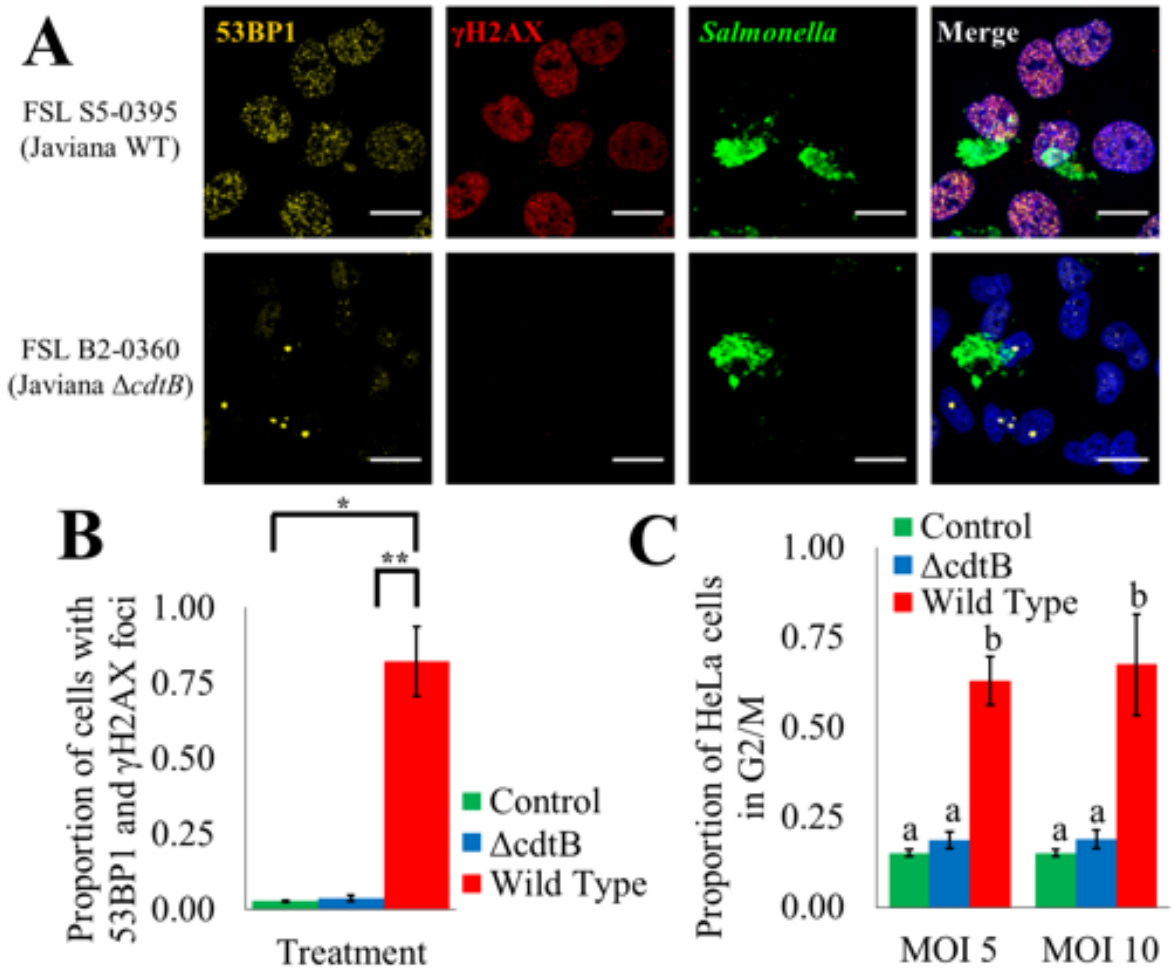

FIGURE 3.5 *cdtB* is essential for activation of the host DNA damage response and G2/M cell cycle arrest. HeLa cells infected with wild-type and Δ*cdtB* isogenic mutants of serotypes Javiana and Montevideo were analyzed for the presence of 53BP1 and γH2AX foci, at 48 hpi. (A) Representative images showing 53BP1 and γH2AX foci, among HeLa cells infected with wild-type Javiana and a Δ*cdtB* isogenic mutant. Scale bars represent 25 µm. (B) Quantification of the proportion of HeLa cells with both 53BP1 and γH2AX foci; data represent 2 technical replicates from 2 independent experiments for each of the 4 strains (Javiana and Montevideo parent strains and their respective isogenic mutants); error bars represent standard deviations. **signifies p-value < 0.01; * signifies p-value < 0.05 (C) Proportion of HeLa cell populations in the G2/M phase determined by DNA content analysis using flow cytometry. HeLa cells were infected at 2 multiplicities of infection with either wild-type or Δ*cdtB* isogenic mutants of serotypes Javiana and Montevideo. Cells were fixed at 48 hpi, and DNA content was measured using PI staining. Data represent the averages for both serotypes from 4 independent experiments for each strain. Bars that do not share identical letters represent statistically different values ($p < 0.05$). Error bars represent standard deviations.

S-CDT-mediated intoxication can occur via autocrine or paracrine pathways.

Immunofluorescence staining clearly showed that cells which were not infected with *Salmonella*, but were located near *Salmonella* infected HeLa cells, had an activated DDR (see Figs 3.3, 3.4,

and 3.5). To determine if the activation of the DDR and G2/M arrest could occur via intoxication arising from paracrine pathways, we co-incubated uninfected HeLa cell populations with cell-free supernatants collected from previous infections with S-CDT-positive serotypes and their respective Δ*cdtB* isogenic mutants. HeLa cell populations incubated with cell-free supernatants from infections with WT isolates encoding S-CDT had a significantly higher proportion of cells in the G2/M phase compared to HeLa cells incubated with cell-free supernatants from Δ*cdtB*-infected HeLa cells, after incubation at 5% ($p = 0.034$) and 20% ($p = 0.014$) total volume (Fig 3.6A).

In contrast, the proportion of HeLa cells in the G2/M phase did not differ significantly between untreated cells, and cells co-incubated with either 5% or 20% v/v cell-free supernatant from a previous infection with Δ*cdtB* isogenic mutants ($p = 0.450$, and $p = 0.979$, for 5% and 20% v/v treatments, respectively).

We also found that HeLa cells co-incubated with filter-sterilized cell-free supernatant from past infections with S-CDT-positive WT strains showed a significantly higher proportion of cells with 53BP1 and γH2AX foci as compared to untreated control cells; this was observed at both concentrations of 5% v/v ($p = 0.047$), and 20% v/v ($p = 0.004$) (Figs 3.6B and 3.6C).

Furthermore, HeLa cells treated with cell-free supernatant from WT infections, had a significantly higher proportion of cells having both 53BP1 and γH2AX foci, compared to cells treated with cell-free supernatant from Δ*cdtB* infections ($p = 0.015$, and $p = 0.003$ for 5% v/v and 20% v/v, respectively). HeLa cells co-incubated with cell-free supernatant from infections with Δ*cdtB* isolates also did not differ significantly from untreated control cells with regard to the proportion of cells with 53BP1 and γH2AX foci ($p = 0.958$ for 5% v/v, $p = 0.555$ for 20% v/v).

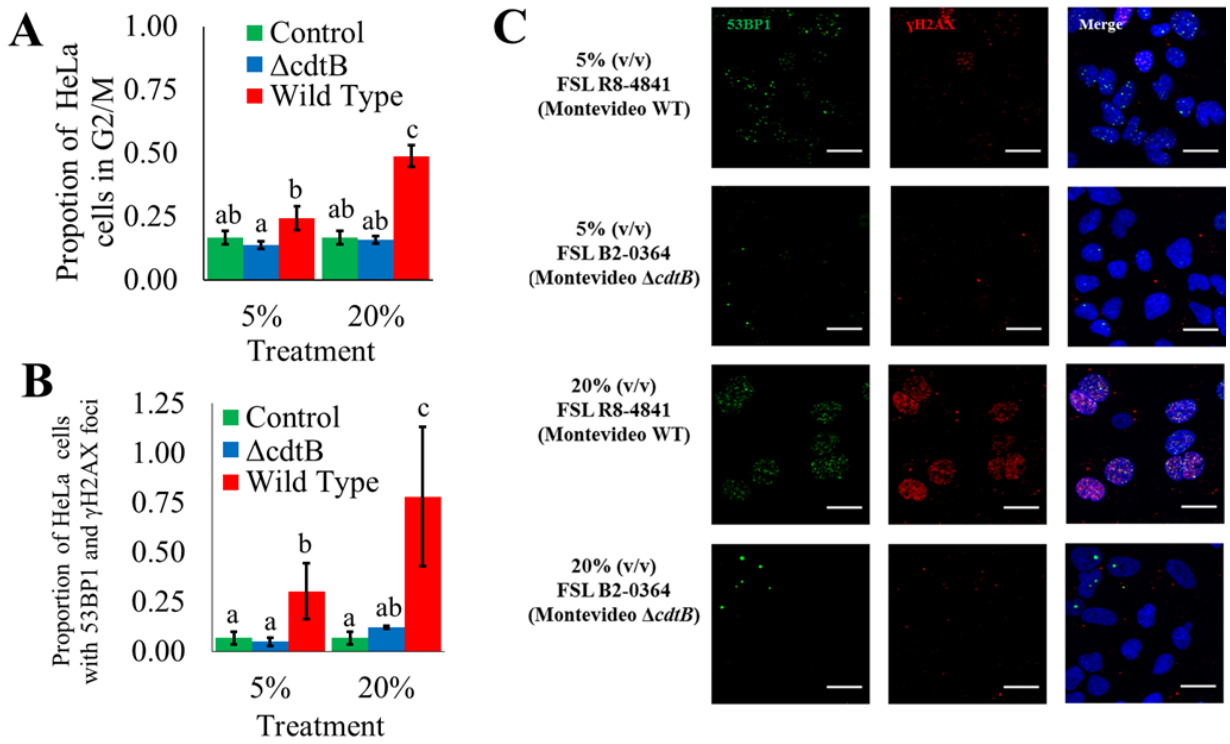

FIGURE 3.6 S-CDT-mediated activation of the DNA damage response and G2/M cell cycle arrest can occur via paracrine pathways. HeLa cells were co-incubated with cell-free supernatants from previous infections with S-CDT-positive wild-type strains FSL S5-0395 (serotype Javiana) and FSL R8-4841 (serotype Montevideo), and their respective isogenic Δ*cdtB* mutants; supernatants were added at final concentrations of 5% and 20% v/v. (A) At 24 h post intoxication, cells were stained with PI to determine DNA content, and quantify the proportion of cells in the G2/M phase. Results represent 2 independent experiments for each of the 4 strains. Bars that do not share identical letters represent statistically different values ($p < 0.05$). (B) HeLa cells grown on coverslips were co-incubated with cell-free supernatants from previous infections. Immunofluorescence staining was performed to detect cells with 53BP1 and γH2AX foci, at 24 h post intoxication. Bar charts represent data from 2 independent experiments. Bars that do not share identical letters represent statistically different values ($p < 0.05$). (C) Representative images are shown for supernatants from infections with FSL R8-4841 (WT strain of serotype Montevideo) and FSL B2-0364 (Δ*cdtB* mutant of serotype Montevideo). Data for FSL S5-0395 (Javiana WT) and FSL B2-0360 (Δ*cdtB* mutant of serotype Javiana) are shown in Fig. S1. Scale bars represent 25 μm.

Heat inactivation of supernatants collected from infection with S-CDT positive isolates abolished the ability of the toxin-containing supernatant to induce 53BP1 and γH2AX foci (see Fig 3.7), suggesting that a heat-labile toxin is responsible for activation of the DDR. This is consistent with previous studies which have shown that heating CDT-containing supernatant from *E. coli*

and *Campylobacter* spp. at 70°C for 1 h abolished the cytotoxic effect of this toxin in tissue culture (36, 37).

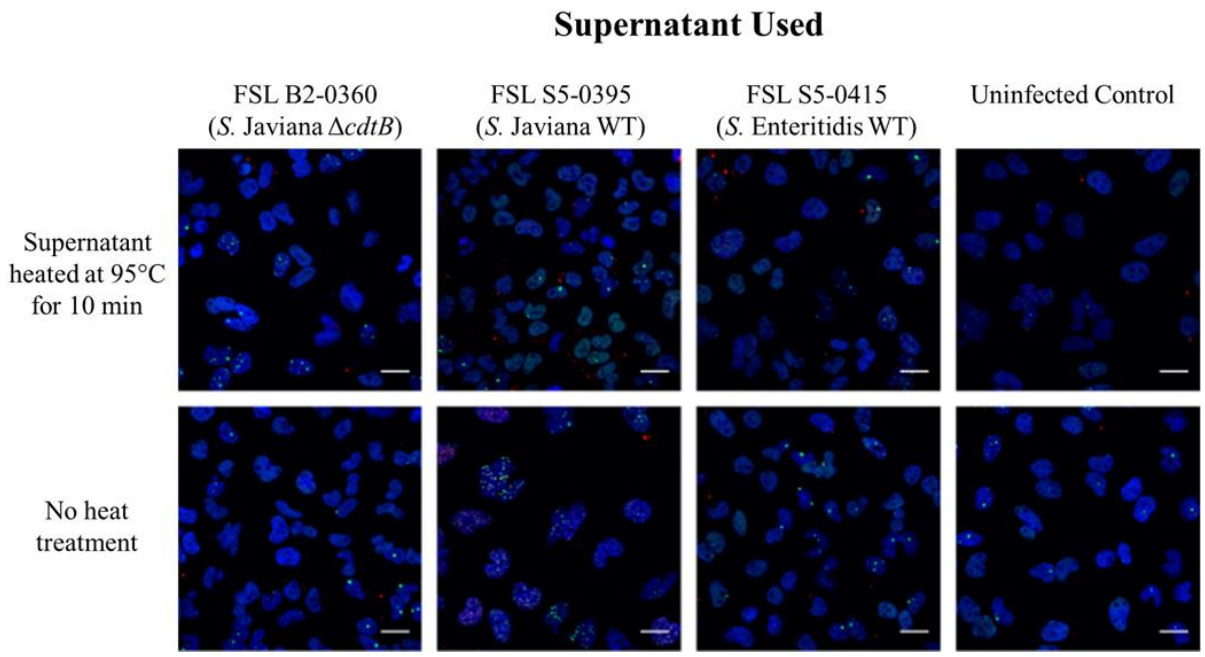

FIGURE 3.7 Heat treatment of supernatants from previous infections inactivates S-CDT-mediated activation of the DNA damage response. Supernatant was collected, at 48 hours post infection, from HeLa cells that were initially infected (or uninfected, in the case of the 'uninfected control') with *Salmonella*; the *Salmonella* Enteritidis WT strain is S-CDT-negative, while the *Salmonella* Javiana WT strain is S-CDT-positive. Supernatants were filtered with a 0.2 μm filter and were subsequently heat treated at 95°C for 10 min. These supernatants were then added (final volume 10% v/v) to HeLa cell cultures, and were incubated for 24 h prior to fixation with 4% PFA. Immunofluorescence staining was performed to detect 53BP1 (green) and γH2AX (red) foci. Nuclei were stained with DAPI. Scale bars represent 25 μm.

Previous studies had suggested a strict requirement for internalization of *Salmonella* cells, in order for S-CDT production to occur (2, 16). Similarly, we found that co-incubation of HeLa cells with either LB broth or EMEM tissue culture media used to culture S-CDT positive *Salmonella*, failed to induce activation of the host DDR, and failed to induce a G2/M arrest (Fig 3.8). This result suggests that the S-CDT production only occurs by intracellular *Salmonella*; hence the paracrine-route of S-CDT-induced intoxication requires initial intracellular infection of at least some host cells to allow for production of S-CDT needed for the paracrine intoxication. Overall, these results suggest that intoxication with S-CDT may occur via autocrine or paracrine pathways, implicating that the eukaryotic cells do not need to be infected with S-CDT-positive NTS in order to sustain DNA damage.

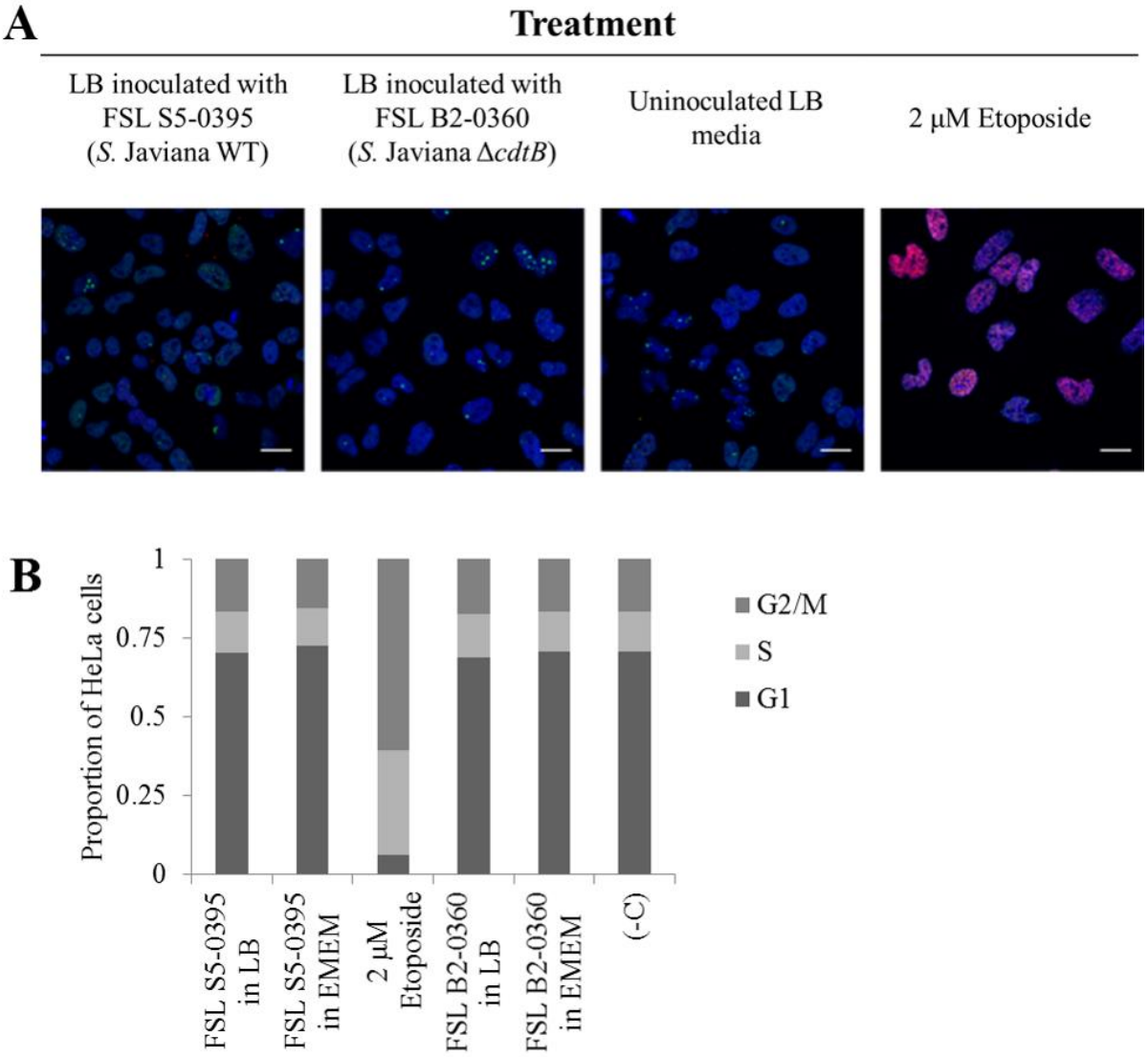

FIGURE 3.8 S-CDT-mediated intoxication does not occur when *Salmonella* are grown in LB broth or in EMEM. (A) *Salmonella* cells were cultured in 0.3 M NaCl LB broth pH 8 at 37°C under stationary conditions until mid-log phase; the LB broth was filtered with a 0.2 μm filter to remove bacterial cells, and the resulting filtered broth (at a final concentration of 10% v/v) was added to HeLa cells grown on glass coverslips in 24-well plates. After 24 h, HeLa cells were fixed with 4% PFA, and immunofluorescence staining was performed to detect γH2AX (red) and 53BP1 foci (green). DAPI is included as a nucleic acid stain. Uninoculated LB was included as a negative control, and 2 μM etoposide was included as a positive control. Scale bars represent 25μm. (B) HeLa cells grown in 6-well plates were co-incubated with sterile-filtered LB broth or EMEM media inoculated with S-CDT-positive *Salmonella* (wild type *S.* Javiana FSL S5-0395) or S-CDT null (Δ*cdtB S.* Javiana FSL B2-0360) at a final concentration of 10% v/v. After 24 h, cells were harvested and subjected to flow cytometry to determine cell cycle phase based on DNA content (i.e. G1, S, or G2/M cell cycle phases).

DISCUSSION

Nontyphoidal *Salmonella* (NTS) serotypes represent one of the most common causes of foodborne illness worldwide, and therefore have a tremendous economic and public health impact. While most NTS infections cause mild gastrointestinal symptoms, systemic infections are not uncommon and may be associated with specific serotypes, such as serotypes Dublin or Choleraesuis (19). In addition, long-term sequelae of NTS infections (e.g., reactive arthritis and post-infectious irritable bowel syndrome) have been reported (38). While recent studies of S-CDT production by *S.* Typhi have suggested that this virulence factor plays an important role in establishing a chronic infection, and in the development of typhoid fever (8, 12), extensive genomic and phenotypic analyses have largely failed to definitively identify specific virulence factors that may be responsible for virulence differences among NTS serotypes, suggesting that multiple virulence factors likely contribute to the severity of an infection (20, 39). The recent identification of genes encoding S-CDT in the genomes of some NTS serotypes, however has provided a specific set of genes that may impart unique virulence characteristics to a subset of NTS serotypes. As the specific contributions of S-CDT to pathogenicity of NTS serotypes have remained largely uncharacterized, we conducted a series of experiments to assess the distribution and conservation of S-CDT-encoding genes among NTS serotypes and to determine the contributions of these genes to *Salmonella* pathogenicity, using a cell culture model.

In this study, we established that among 21 NTS serotypes causing the majority of human clinical cases of salmonellosis in the US, serotypes Javiana, Montevideo, Oranienburg, as well as some serotype Mississippi isolates, encode S-CDT. Interestingly, we found that serotype Mississippi is polyphyletic, and that S-CDT-encoding genes are only present for isolates in 1 of the 2 clades. We estimated that infections with S-CDT-positives isolates representing these serotypes accounts for approximately 150,280 cases (90% C.I.: 111,812 – 196,954) of foodborne salmonellosis in the US in 2011, suggesting that a relatively large population of individuals are exposed to this toxin (17). In general, the results of our PCR screen agree with previous reports on the distribution of S-CDT-encoding genes among NTS serotypes (20, 22-24). For example,

using a much smaller set of isolates (1 – 2 isolates) per serotype, den Bakker *et al.* also reported that serotypes Javiana, Montevideo, Oranienburg encode *cdtB* (22). While our study focused on characterizing S-CDT presence among the most common NTS serotypes causing human salmonellosis in the US, other studies indicated that in addition to serotypes Javiana, Montevideo, Oranienburg, and Mississippi, at least 37 other, rarer, NTS serotypes also encode S-CDT, suggesting further human exposure to S-CDT-positive NTS strains (22, 25). While some evidence supports that S-CDT-positive NTS represent a specific NTS clade, there is at least some evidence that S-CDT is not exclusively associated with a specific clade (22). For example, a recent study by den Bakker *et al.* identified that *Salmonella* serotypes encoding S-CDT were primarily found in Clade B (e.g., serotypes Javiana, Schwarzengrund, Montevideo), while a small sub-clade (including serotypes Paratyphi A and Typhi) of Clade A also represented strains that encode S-CDT (22). While future additional studies on the presence of S-CDT among additional NTS serotypes will be valuable, the data presented here on S-CDT presence among a large number of isolates representing serotypes commonly associated with human disease provides important information that allows for an initial assessment of the population in the US which is exposed to S-CDT through infections involving NTS serotypes.

Not surprisingly, alignments of predicted amino acid sequences suggested that CdtB, PltA, and PltB are highly conserved across serotypes, with the active subunit (CdtB) having the fewest amino acid substitutions. It is interesting to note that several SNPs in the coding sequences of PltB and PltA involved transitions to codons which are less frequently used (i.e. "rare") by *Salmonella*, potentially leading to a reduction in the overall quantities of these proteins in some strains (40). Importantly, our data suggest that the S-CDT produced by NTS serotypes, based on overall nucleotide similarity to coding sequences of S-CDT genes encoded by *S.* Typhi, likely induces DNA damage in eukaryotic cells in a similar manner to the S-CDT produced by *S.* Typhi.

To date, few studies have specifically examined the contributions of S-CDT production by NTS serotypes to infection and pathogenesis (23-25). While limited prior studies have shown

that some S-CDT-positive serotypes (e.g., Javiana) induce a G2/M arrest (23, 25), our results not only confirmed that infection with S-CDT-positive NTS serotypes causes a cell cycle arrest that is not observed in cells infected with S-CDT-negative serotypes, but also show that this cell cycle arrest is associated with activation of the host DDR. In addition, we show that serotypes Oranienburg and Mississippi also induce DNA damage in infected eukaryotic cells, further confirming that an active S-CDT is produced by these NTS serotypes, and that the DNA damaging effects are not restricted to serotype Typhi. Our data show that infection with wild-type strains of S-CDT-positive NTS serotypes significantly impacts the cellular outcome of infection, suggesting that S-CDT is an important pathogenicity factor in strains that encode S-CDT.

An important difference between S-CDT and the CDTs produced by other Gram-negative pathogens is the additional ADP-ribosyl transferase activity of the PltA subunit in S-CDT (2, 3). Here we confirmed that the DNA damaging effects are due to the activity of the CdtB subunit, as deletion of *cdtB* abolished the ability of S-CDT-positive serotypes to cause DNA damage (as shown by the G2/M cell cycle arrest and the 53BP1 and γH2AX foci). This is in agreement with previous studies in serotype Typhi, which also showed that *cdtB* deletion abolishes G2/M cell cycle arrest (2, 16). We therefore conclude that the S-CDT produced by NTS acts in the same manner as the S-CDT produced by serotype Typhi.

Immunofluorescence staining of HeLa cells infected with S-CDT-positive NTS isolates showed that the DNA damage sustained from S-CDT-mediated intoxication is not restricted to infected cells, but impacts nearby cells as well. Previous studies with *S.* Typhi have similarly shown that intoxication with S-CDT may occur via autocrine or paracrine pathways, as intoxication of Henle cells in the presence of a toxin-neutralizing antibody abolished the cell cycle arrest of cells co-incubated with cell-free supernatant containing S-CDT from previous infections (2). In support of this, a previous study also showed that exogenous addition of purified S-CDT was sufficient to cause a G2/M phase arrest, suggesting that S-CDT is necessary and sufficient for the cytotoxic activity observed in S-CDT-encoding serotypes of *Salmonella*

(12). Furthermore, Guidi *et al.* (2013) showed that S-CDT is transported anterograde along host microtubules of infected cells in outer membrane vesicles and secreted from infected cells into the surrounding media (41). Consequently, it is likely that all cells, whether or not they are infected with *Salmonella*, sustain S-CDT-mediated DNA damage via the same mechanism, as all cells appear to take up S-CDT from the extracellular environment. This is important because during an infection, relatively few *S. enterica* cells successfully invade host cells (42). Together, these results suggest that dissemination of S-CDT into the surrounding tissues has the potential to impact a greater number of host cells, which may contribute to the overall outcome of infection.

It has been established previously that *Salmonella* may induce host cell death via multiple mechanisms, including apoptosis and pyroptosis, depending on the host cell type (*e.g.,* immune versus epithelial cells) (43-45). The majority of these analyses have been conducted using serotype Typhimurium, which is now known to be S-CDT negative. Therefore, S-CDT-mediated intoxication represents an important new mechanism by which NTS may induce cell death. While HeLa cells represent a standard cell line used for studies of DNA damage, future studies examining the cellular outcome of infection with S-CDT positive NTS serotypes in non-cancerous (i.e. non-transformed) cell lines will be important to assess whether eukaryotic cells are able to repair the DNA damage induced by S-CDT positive NTS serotypes.

Our data indicate that S-CDT significantly impacts the outcome of infection with NTS serotypes at the cellular level. It will be important to further assess the role of S-CDT *in vivo* to determine the implications of S-CDT-mediated DNA damage at the host level, and to also ascertain what, if any, long-term sequelae may result from the DNA damage sustained during an infection with S-CDT producing NTS. An analysis by Rodriguez-Rivera *et al*. (2015) reported that nontyphoidal serotypes which had a higher proportion of infections resulting in invasive disease (as reported by Jones *et al*. (2010) were significantly more likely to encode S-CDT, therefore suggesting that S-CDT contributes to the severity of human clinical cases as well (19, 25). AbuOun et al. (2005) found that sera from humans who had previously been exposed to *C.*

jejuni (which encodes a CDT) neutralized the activity of the toxin *in vitro*, suggesting that individuals seroconvert following exposure to CDTs (46). Further studies will be beneficial in determining if S-CDT exposure also causes antibody production, and potentially neutralization, of S-CDT produced by NTS.

Overall, the data provided here contribute important new information that strongly suggest that a subset of NTS strains (i.e., those encoding S-CDT) are likely to cause unique consequences of infection at both the cellular and host level. Hence, there is an urgent need to determine the potential public health impacts of S-CDT-producing NTS serotypes. Ultimately, this work may lead to differential assessment of the food safety hazards posed by foods contaminated with S-CDT-positive and negative NTS strains.

ACKNOWLEDGEMENTS

The authors would like to thank Carol Bayles and the Cornell Bio-Imaging Facility for their invaluable assistance with the flow cytometry and microscopic analyses. The authors would also like to thank Dr. Lynn Johnson for her guidance with the statistical analyses. R.M. was supported by USDA NIFA award 2016-67011-24714. Research on *Salmonella* in the Wiedmann lab is currently supported by the National Institute of Food and Agriculture, USDA Hatch Grant NYC-143436. The Bio-Imaging Facility at Cornell University is supported by NIH Grant 1S10RR025502.

REFERENCES

1. Gargi A, Reno M, Blanke SR. 2012. Bacterial toxin modulation of the eukaryotic cell cycle: are all cytolethal distending toxins created equally? Frontiers in cellular and infection microbiology 2.
2. Spanò S, Ugalde JE, Galán JE. 2008. Delivery of a *Salmonella* Typhi exotoxin from a host intracellular compartment. Cell host & microbe 3:30-38.
3. Jinadasa RN, Bloom SE, Weiss RS, Duhamel GE. 2011. Cytolethal distending toxin: a conserved bacterial genotoxin that blocks cell cycle progression, leading to apoptosis of a broad range of mammalian cell lineages. Microbiology 157:1851-1875.
4. Guerra L, Cortes-Bratti X, Guidi R, Frisan T. 2011. The biology of the cytolethal distending toxins. Toxins 3:172-190.
5. DiRienzo JM. 2014. Uptake and Processing of the Cytolethal Distending Toxin by Mammalian Cells. Toxins 6:3098-3116.
6. Schultz LB, Chehab NH, Malikzay A, Halazonetis TD. 2000. p53 binding protein 1 (53BP1) is an early participant in the cellular response to DNA double-strand breaks. The Journal of cell biology 151:1381-1390.
7. Sharma A, Singh K, Almasan A. 2012. Histone H2AX phosphorylation: a marker for DNA damage. Methods Mol Biol 920:613-626.
8. Belluz LDB, Guidi R, Pateras IS, Levi L, Mihaljevic B, Rouf SF, Wrande M, Candela M, Turroni S, Nastasi C. 2016. The Typhoid Toxin Promotes Host Survival and the Establishment of a Persistent Asymptomatic Infection. PLoS Pathog 12:e1005528.
9. Ge Z, Feng Y, Whary MT, Nambiar PR, Xu S, Ng V, Taylor NS, Fox JG. 2005. Cytolethal distending toxin is essential for *Helicobacter hepaticus* colonization in outbred Swiss Webster mice. Infection and immunity 73:3559-3567.
10. Ge Z, Rogers AB, Feng Y, Lee A, Xu S, Taylor NS, Fox JG. 2007. Bacterial cytolethal distending toxin promotes the development of dysplasia in a model of microbially induced hepatocarcinogenesis. Cellular microbiology 9:2070-2080.
11. Nešić D, Hsu Y, Stebbins CE. 2004. Assembly and function of a bacterial genotoxin. Nature 429:429-433.
12. Song J, Gao X, Galán JE. 2013. Structure and function of the *Salmonella* Typhi chimaeric A2B5 typhoid toxin. Nature 499:350-354.
13. Locht C, Coutte L, Mielcarek N. 2011. The ins and outs of pertussis toxin. Febs j 278:4668-4682.
14. Paton AW, Paton JC. 2010. *Escherichia coli* Subtilase Cytotoxin. Toxins 2:215-228.
15. Parkhill J, Dougan G, James K, Thomson N, Pickard D, Wain J, Churcher C, Mungall K, Bentley S, Holden M. 2001. Complete genome sequence of a multiple drug resistant Salmonella enterica serovar Typhi CT18. Nature 413:848-852.
16. Haghjoo E, Galán JE. 2004. *Salmonella* Typh iencodes a functional cytolethal distending toxin that is delivered into host cells by a bacterial-internalization pathway. Proceedings of the National Academy of Sciences of the United States of America 101:4614-4619.
17. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson M-A, Roy SL, Jones JL, Griffin PM. 2011. Foodborne illness acquired in the United States—major pathogens. Emerg Infect Dis 17.
18. Majowicz SE, Musto J, Scallan E, Angulo FJ, Kirk M, O'Brien SJ, Jones TF, Fazil A, Hoekstra RM. 2010. The global burden of nontyphoidal *Salmonella* gastroenteritis. Clinical Infectious Diseases 50:882-889.

19. Jones TF, Ingram LA, Cieslak PR, Vugia DJ, Tobin-D'Angelo M, Hurd S, Medus C, Cronquist A, Angulo FJ. 2008. Salmonellosis outcomes differ substantially by serotype. Journal of Infectious Diseases 198:109-114.
20. Suez J, Porwollik S, Dagan A, Marzel A, Schorr YI, Desai PT, Agmon V, McClelland M, Rahav G, Gal-Mor O. 2013. Virulence gene profiling and pathogenicity characterization of non-typhoidal *Salmonella* accounted for invasive disease in humans. PLoS One 8:e58449.
21. Sabbagh SC, Forest CG, Lepage C, Leclerc J-M, Daigle F. 2010. So similar, yet so different: uncovering distinctive features in the genomes of Salmonella enterica serovars Typhimurium and Typhi. FEMS microbiology letters 305:1-13.
22. den Bakker HC, Switt AIM, Govoni G, Cummings CA, Ranieri ML, Degoricija L, Hoelzer K, Rodriguez-Rivera LD, Brown S, Bolchacova E. 2011. Genome sequencing reveals diversification of virulence factor content and possible host adaptation in distinct subpopulations of *Salmonella enterica*. BMC genomics 12:425.
23. Williams K, Gokulan K, Shelman D, Akiyama T, Khan A, Khare S. 2015. Cytotoxic mechanism of cytolethal distending toxin in nontyphoidal salmonella serovar (*Salmonella* Javiana) during macrophage infection. DNA and cell biology 34:113-124.
24. Mezal EH, Bae D, Khan AA. 2014. Detection and functionality of the CdtB, PltA, and PltB from *Salmonella enterica* serovar Javiana. Pathogens and disease 72:95-103.
25. Rodriguez-Rivera LD, Bowen BM, den Bakker HC, Duhamel GE, Wiedmann M. 2015. Characterization of the cytolethal distending toxin (typhoid toxin) in non-typhoidal *Salmonella* serovars. Gut pathogens 7:1.
26. Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A 97:6640-6645.
27. Lee CA, Falkow S. 1990. The ability of *Salmonella* to enter mammalian cells is affected by bacterial growth state. Proceedings of the National Academy of Sciences 87:4304-4308.
28. Galán JE, Curtiss R. 1990. Expression of *Salmonella* Typhimurium genes required for invasion is regulated by changes in DNA supercoiling. Infection and immunity 58:1879-1885.
29. Hassan JO, Curtiss R. 1996. Effect of vaccination of hens with an avirulent strain of *Salmonella* Typhimurium on immunity of progeny challenged with wild-type *Salmonella* strains. Infection and Immunity 64:938-944.
30. Achtman M, Wain J, Weill F-X, Nair S, Zhou Z, Sangal V, Krauland MG, Hale JL, Harbottle H, Uesbeck A. 2012. Multilocus sequence typing as a replacement for serotyping in *Salmonella enterica*. PLoS Pathog 8:e1002776.
31. Wersto RP, Chrest FJ, Leary JF, Morris C, Stetler-Stevenson M, Gabrielson E. 2001. Doublet discrimination in DNA cell-cycle analysis. Cytometry 46:296-306.
32. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B. 2012. Fiji: an open-source platform for biological-image analysis. Nature methods 9:676-682.
33. Toller IM, Neelsen KJ, Steger M, Hartung ML, Hottiger MO, Stucki M, Kalali B, Gerhard M, Sartori AA, Lopes M. 2011. Carcinogenic bacterial pathogen *Helicobacter pylori* triggers DNA double-strand breaks and a DNA damage response in its host cells. Proceedings of the National Academy of Sciences 108:14944-14949.
34. Fedor Y, Vignard J, Nicolau-Travers ML, Boutet-Robinet E, Watrin C, Salles B, Mirey G. 2013. From single-strand breaks to double-strand breaks during S-phase: a new mode of action of the *Escherichia coli* Cytolethal Distending Toxin. Cell Microbiol 15:1-15.

35. Mochan TA, Venere M, DiTullio RA, Halazonetis TD. 2004. 53BP1, an activator of ATM in response to DNA damage. DNA repair 3:945-952.
36. Johnson W, Lior H. 1987. Response of Chinese hamster ovary cells to a cytolethal distending toxin (CDT) of *Escherichia coli* and possible misinterpretation as heat-labile (LT) enterotoxin. FEMS microbiology letters 43:19-23.
37. Johnson W, Lior H. 1988. A new heat-labile cytolethal distending toxin (CLDT) produced by *Escherichia coli* isolates from clinical material. Microbial pathogenesis 4:103-113.
38. Connor BA, Riddle MS. 2013. Post-infectious sequelae of travelers' diarrhea. Journal of travel medicine 20:303-312.
39. de Jong HK, Parry CM, van der Poll T, Wiersinga WJ. 2012. Host–pathogen interaction in invasive salmonellosis.
40. Gustafsson C, Govindarajan S, Minshull J. 2004. Codon bias and heterologous protein expression. Trends in biotechnology 22:346-353.
41. Guidi R, Levi L, Rouf SF, Puiac S, Rhen M, Frisan T. 2013. *Salmonella enterica* delivers its genotoxin through outer membrane vesicles secreted from infected cells. Cellular microbiology 15:2034-2050.
42. Ackermann M, Stecher B, Freed NE, Songhet P, Hardt W-D, Doebeli M. 2008. Self-destructive cooperation mediated by phenotypic noise. Nature 454:987-990.
43. Fink SL, Cookson BT. 2007. Pyroptosis and host cell death responses during *Salmonella* infection. Cell Microbiol 9:2562-2570.
44. Boise LH, Collins CM. 2001. *Salmonella*-induced cell death: apoptosis, necrosis or programmed cell death? Trends in microbiology 9:64-67.
45. Knodler LA, Finlay BB, Steele-Mortimer O. 2005. The *Salmonella* effector protein SopB protects epithelial cells from apoptosis by sustained activation of Akt. Journal of Biological Chemistry 280:9058-9064.
46. AbuOun M, Manning G, Cawthraw SA, Ridley A, Ahmed IH, Wassenaar TM, Newell DG. 2005. Cytolethal distending toxin (CDT)-negative *Campylobacter jejuni* strains and anti-CDT neutralizing antibodies are induced during human infection but not during colonization in chickens. Infection and immunity 73:3053-3062.

CHAPTER 4

TWO IS BETTER THAN ONE: NONTYPHOIDAL *SALMONELLA* SEROTYPE JAVIANA ENCODES MULTIPLE BINDING SUBUNITS OF THE SALMONELLA CYTOLETHAL DISTENDING TOXIN FOR ENHANCED ACTIVITY *IN VITRO*

ABSTRACT

The ‘typhoid toxin’ (also known as the Salmonella cytolethal distending toxin [S-CDT]) produced by *Salmonella enterica* subsp. *enterica* serotype Typhi (*S.* Typhi) acts a nuclease *in vitro*, and is hypothesized to play a crucial role in the development of typhoid fever. Recently, it was found that >40 nontyphoidal serotypes also encoded S-CDT. S-CDT is encoded on two operons, and includes genes encoding toxin subunits (*pltA*, *pltB*, *cdtB*), as well as two genes, *ttsA*, encoding a putative phage muramidase, and *STY1887*, which has an unknown function. The requirement of each gene for S-CDT-mediated cytotoxicity *in vitro* had not yet been characterized for nontyphoidal serotypes. To this end, we constructed *S.* Javiana strains harboring deletions in each gene in the S-CDT islet. Infection of the normal human intestinal epithelial cells (HIEC-6) revealed that *pltA* and *cdtB* are essential for S-CDT-mediated DNA damage, similar to what had been shown for *S.* Typhi. While *STY1887* was also dispensable for S-CDT activity *in vitro*, we found that, in contrast to what had been shown for *S.* Typhi, *pltB* and *ttsA* are also dispensable as infections with these strains did not result in a significant activation of the DNA damage response (DDR) in HIEC-6 cells. Simultaneous deletion of both *pltB,* and its homolog *artB,* abolished activity, suggesting that *S.* Javiana encodes two variants of the binding subunit, which could be important for differences in targeting host cells. Overall, these results suggest that production of active S-CDT has different genetic requirements in typhoidal and nontyphoidal serotypes.

IMPORTANCE

Nontyphoidal *Salmonella* is the second leading bacterial cause of foodborne illness world-wide. The recent discovery of the ‘typhoid toxin’ in select nontyphoidal serotypes represents an important gap in our understanding of nontyphoidal salmonellosis. Here, we show that genes encoding the active subunit, CdtB, and the ADP-ribosylation subunit, PltA, are

essential for S-CDT-mediated activation of the DNA damage response in normal human epithelial cells. However, the binding subunit was not essential, as infection with these strains resulted in an altered cell cycle progression, and had DDR foci indicative of DNA damage, similar to infection with wild-type bacteria. These data provide important information regarding the function of the typhoid toxin in NTS. Furthermore, our results show that ArtB may substitute for PltB *in vitro*, which may enhance production of the complete holotoxin, as both binding subunits are required for maximum S-CDT-mediated cytotoxicity.

INTRODUCTION

Infections by nontyphoidal *Salmonella* (NTS) serotypes account for an estimated 93.8 million illnesses, and 155,000 deaths per year (1), making it the second leading cause of bacterial foodborne disease worldwide (2). The discovery of a cytolethal distending toxin (CDT) in *S. enterica* subsp. *enterica* serotype Typhi, the causative agent of typhoid fever, led to the toxin being named the 'typhoid toxin' (3, 4). However, genomic screens have shown that at least 40 NTS serotypes encode genes for the 'typhoid toxin' as well (5-7), and further *in vitro* characterizations have shown that these toxin positive serotypes produce an active toxin (6, 8, 9).

The CDT encoded by *Salmonella* differs from the CDT produced by other Gram-negative pathogens. In all other Gram-negative pathogens, CDT is encoded by 3 genes; *cdtA* and *cdtC* encode subunits CdtA and CdtC which play a role in toxin binding and trafficking within eukaryotic cells (10, 11), while *cdtB* encodes the active subunit CdtB, which is predicted to act as a nuclease (12). While *Salmonella* encodes *cdtB*, it does not encode *cdtA* or *cdtC* (5, 13). Instead, the CDT produced by *Salmonella* uses subunits PltA and PltB, which are homologous to the S1 and S2 subunits of the pertussis toxin, for binding and trafficking (4). For these reasons, we will refer to the special variant of CDT as S-CDT (for *Salmonella* CDT).

In vitro, infection with S-CDT positive strains of toxin positive serotypes (e.g., Typhi, Javiana, Montevideo, Mississippi, and Schwarzengrund) results in a G2/M cell cycle arrest (3, 6, 9, 13), and activation of the DNA damage response (DDR) as determined by immunofluorescence staining for DDR proteins γH2AX and 53BP1 (8). *In vivo*, administration of purified S-CDT partially re-capitulated symptoms of typhoid fever in a mouse model (4). Similarly, infection with S-CDT-carrying strains of *S.* Typhimurium (a naturally S-CDT toxin negative serotype (8)) prolonged carriage of toxin positive strains, suggesting that the toxin alters the host-pathogen interaction *in vivo*, enabling S-CDT-positive *Salmonella* to persist for longer periods of time in the host (14).

S. enterica subsp. *enterica* serotype Javiana is the 4th most commonly isolated NTS serotype causing infections in the U.S. (15), and is the most common S-CDT-positive serotype. The majority of studies characterizing S-CDT activity have been performed using serotype Typhi, which has several important genotypic and phenotypic differences from NTS serotypes (16-18). While the roles of the other two accessory genes in the S-CDT islet, *STY1887* and *ttsA*, have been characterized in Typhi (19), their role in the secretion and production of S-CDT in NTS serotypes, has not been established. Therefore, characterization of S-CDT in NTS serotypes represents a knowledge gap in our understanding of the role of S-CDT in NTS infections.

Previous characterizations *in vitro*, which were critical for asserting the activity of S-CDT, were conducted using cancer cell lines, and therefore limited analyses to determining whether or not DNA damage had occurred. Use of a non-transformed cell line is therefore necessary to understand how infection with S-CDT-positive *Salmonella* impacts the cellular outcome of infection.

To determine the role of all genes in the S-CDT islet on S-CDT activity in NTS serotype *S.* Javiana, we infected normal human intestinal epithelial cells with strains of *S.* Javiana harboring gene deletions in different genes in the S-CDT islet. We found that infection of normal cells also results in a G2/M cell cycle arrest, and activation of the DDR. Genes *cdtB* and *pltA*, are essential for toxin activity *in vitro*, while *pltB, STY1887,* and *ttsA* are not. Moreover, we show that *S.* Javiana encodes multiple subunits, *pltB* and *artB,* which can be used interchangeably *in vitro*, for enhanced activity of S-CDT.

RESULTS

Infection with S-CDT-positive *S.* Javiana induces a DDR in normal epithelial cells. We previously established in a HeLa cell model, that infection with S-CDT positive strains of NTS activated the DDR and induced a G2/M arrest, while infection with either S-CDT negative serotypes or Δ*cdtB* mutants did not (8). To determine if non-cancerous cells were also susceptible to S-CDT intoxication, we infected normal human intestinal epithelial cells (HIEC-6 cells) with wild-type and Δ*cdtB* strains of *S.* Javiana. Infection with wild-type *S.* Javiana resulted in 44% of cells with 53BP1 and γ-H2AX foci, compared to just 6% in either uninfected cells, or in cell populations infected with the *ΔcdtB* strain (P = 0.001; Fig. 4.1A and 4.1B). Similarly, HIEC-6 cell populations infected with wild-type *S.* Javiana also had significantly higher proportions of cells in the G2/M phase (P = 0.005) compared to uninfected controls (Fig. 4.1C). Cell populations infected with wild-type *S.* Javiana had 26% of cells in the G2/M phase, compared to 12% of cells in G2/M among uninfected controls. Similar to uninfected controls, infection with Δ*cdtB S.* Javiana resulted in cell populations having 11% of cells in G2/M, suggesting that the interference in cell cycle progression is dependent on the *Salmonella* encoding *cdtB*.

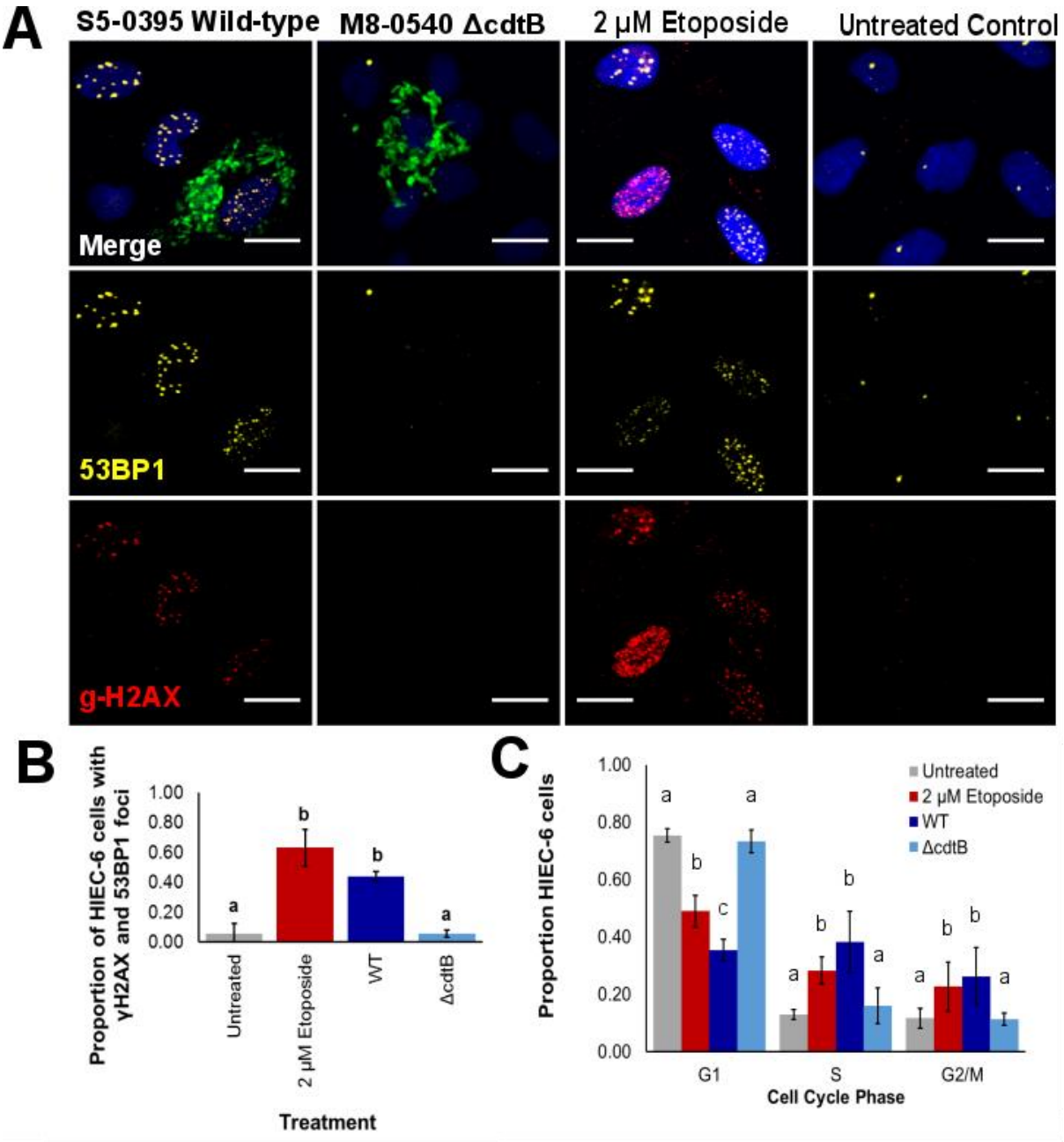

FIGURE 4.1 Infection with wild-type *S.* Javiana activates the DDR and induces a G2/M phase arrest. Normal human intestinal epithelial cells (HIEC-6 cells) were infected with 2 x 10^6 or 4 x 10^6 *S.* Javiana cells for immunofluorescence or flow cytometry analyses, respectively. As a positive control, cells were treated with the topoisomerase poison Etoposide (at a final concentration of 2 μM) for 24 h. At 48 hpi, cells were collected and stained for DDR foci γH2AX and 53BP1, or were stained with propidium iodide for flow cytometry analyses. A) Representative images of cells infected with wild-type *S.* Javiana and Δ*cdtB* strains, and positive (treated with 2 μM etoposide) and negative (untreated) controls. B) Quantification of proportions of cells with >four 53BP1 foci, which co-localized with γH2AX foci. Treatments which do not share letters have significantly different ($p < 0.05$) proportions of cells with 53BP1 foci that also co-localized with γH2AX foci. C) Cells were stained with PI to determine DNA content, and the proportion of cell populations in each cell cycle phase. Treatments that do not share letters within each cell cycle phase group (i.e., G1, S, G2/M) are significantly different ($p < 0.05$).

***pltA* and *cdtB*, but not *pltB*, *ttsA*, or *STY1887* are required for S-CDT activity *in vitro*.** S-CDT is encoded in two operons (Fig 4.2B.). In addition to genes encoding S-CDT (*pltB*, *pltA*, and *cdtB*), the islet also contains a putative bacteriophage muramidase, *ttsA*, which is hypothesized to play a role in toxin secretion (19), and *STY1887* which has an unknown function. To determine which genes were essential for S-CDT-mediated intoxication of HIEC-6 cells, we constructed strains with deletions in individiual genes in the S-CDT islet.

Infection with strains harboring deletions in *cdtB* and *pltA* did not induce DDR foci when these mutants were used to infect HIEC-6 cells (Fig. 4.2A and 4.2C); HIEC-6 cells infected with Δ*cdtB* or Δ*pltA* strains had an average of 5.4% and 7.6% of cells that were double positive for DDR foci 53BP1 and γH2AX; infection with these strains did not differ significanlty from uninfected controls (infection with Δ*cdtB* vs. control P = 1.000 and Δ*pltA* vs. control P = 0.818,). Deletion of *ttsA* resulted in slightly lower levels of HIEC-6 cells with DDR foci, compared to wild-type *S.* Javiana (e.g., 31.6% of cells, versus 43.9%), but this was not significantly different (P = 1.000). *ttsA* is essential for S-CDT-mediated intoxication for *S.* Typhi, in contrast to *S.* Javiana.

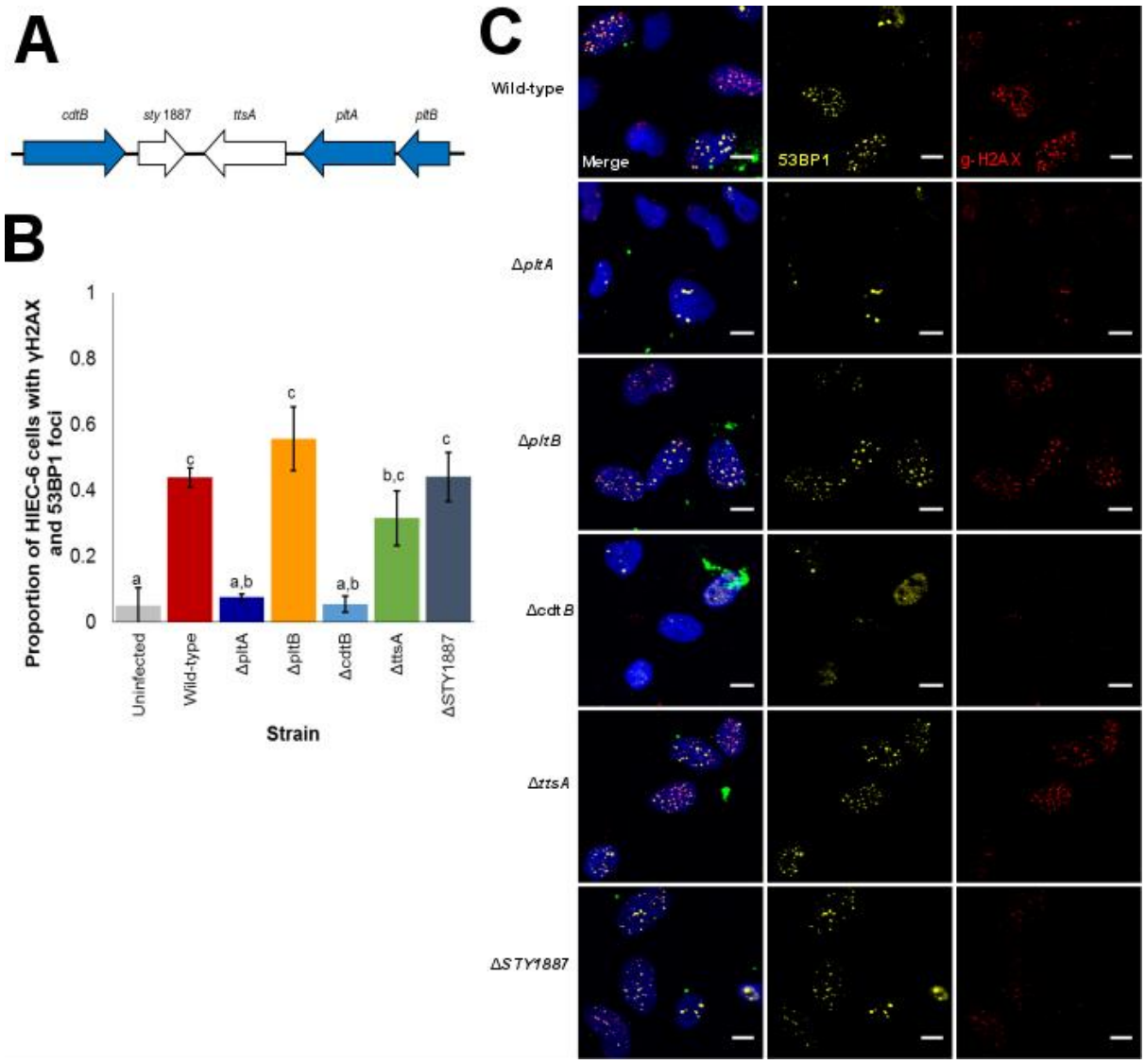

FIGURE 4.2 *pltB, ttsA,* and *STY1887* are not required for S-CDT-mediated intoxication of HIEC-6 cells. Normal human intestinal epithelial cells (HIEC-6 cells) were infected with 2 x 10^6 CFU of *S.* Javiana strains harboring deletions in various genes in the S-CDT islet. Immunofluorescence staining was performed for DDR foci γH2AX and 53BP1 as described above. A) Organization of the S-CDT islet in *S.* Javiana. Genes shown in blue represent genes encoding S-CDT subunits; genes shown in white are contained within the islet, but do not encode protein products that become part of the S-CDT holotoxin. B) Quantification of proportions of cells with >four 53BP1 foci, which co-localized with γH2AX foci. Treatments which do not share letters have significantly different ($p < 0.05$) proportions of cells with 53BP1 foci that also co-localized with γH2AX foci. C) Representative images of cells infected with wild-type *S.* Javiana and S-CDT single gene deletion strains. Data for wild-type and Δ*cdtB* strains are included for ease of comparison, but are also represented in Fig. 1.

To determine if this discrepancy could be due to highly divergent sequences of *ttsA* in *S.* Typhi (strains CT-18 and Ty2) compared to *ttsA* encoded by S-CDT-positive nontyphoidal serotypes, we aligned the sequences of *ttsA* from typhoidal (e.g., Typhi strains CT-18 and Ty2, and Paratyphi A strain ATCC 11511) and nontyphoidal strains (e.g., Javiana, Montevideo, and Schwarzengrund). *ttsA* is highly conserved (Fig 4.3), with just 2 out of 180 amino acids different among the serotypes included, suggesting that either these 2 amino acid residues are essential for activity, or that *ttsA* is not required for S-CDT export in NTS serotypes.

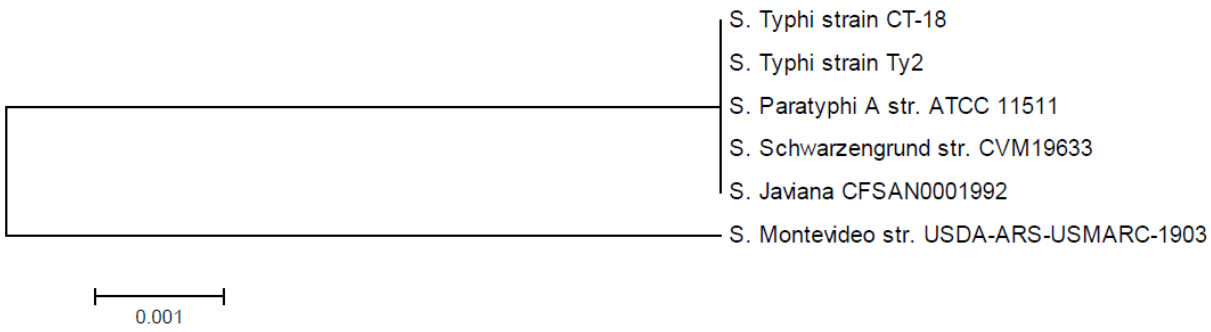

FIGURE 4.3 Amino acid alignment of TtsA from typhoidal and nontyphoidal serotypes. Amino acid sequence was predicted from *artB* equences extracted from whole genome sequence data. A maximum likelihood tree constructed based on translated sequence data for *artB*, encoding using WAG method with gamma distributed sites and 1000 bootstrap repetitions. Bar represents 0.001 substitutions per site.

Strains harboring deletions in *STY1887* or *pltB* resulted in DDR foci in an average of 44.1% and 55.7% of HIEC-6 cells, respectively, and did not differ significantly from proportions of HIEC-6 cells with DDR foci among cells infected with wild-type *S.* Javiana (*STY1887* vs wild-type = 1.000 , Δ*pltB* vs. wild-type P = 0.779), suggesting that these genes are not essential for S-CDT-mediated intoxication.

***S.* Javiana encodes a *pltB* homolog, *artB*, which can substitute for PltB *in vitro*.** Because deletion of *pltB*, encoding the binding subunit of S-CDT, did not have a significant effect on S-CDT-induced intoxication of infected HIEC-6 cells, we hypothesized that *artB*, encoding a homologous protein ArtB (20), could potentially substitute for PltB *in vitro*. *S.* Javiana encodes a truncated version of *artA* and a full-length copy of *artB* (Fig. 4.4A). To determine the importance of *artA* and *artB* in S-CDT intoxication, we constructed strains with deletions in (i) the entire *artAB* operon, or (ii) just *artB* (Fig. 4.4A). HIEC-6 cells infected with either Δ*artAB* or Δ*artB* had an average of 25.4% and 21.0% of cells with DDR foci (Fig. 4.4B), which did not differ from HIEC-6 cell populations infected with either wild-type or Δ*pltB* strains (P > 0.2 for all pairwise comparisons of wild-type, Δ*pltB*, Δ*artB*, Δ*artAB*). However, deletion of both *pltB* and *artB*, abolished S-CDT's ability to induce DDR foci in HIEC-6 cells (Fig. 4.4B and 4.4C). Compared to HIEC-6 cells infected with Δ*pltB* which had an average of 55.7% of cells with DDR foci, infection with Δ*pltB* Δ*artB* resulted in an average of 3.9% of cells with DDR foci (P< 0.001; Fig. 4.4B and 4.4C). Similarly, infection with Δ*pltB* Δ*artAB* resulted in a significantly lower level of HIEC-6 cells with evidence of S-CDT intoxication (5.2% of cells with DDR foci, P <0.0001 compared to wild-type).

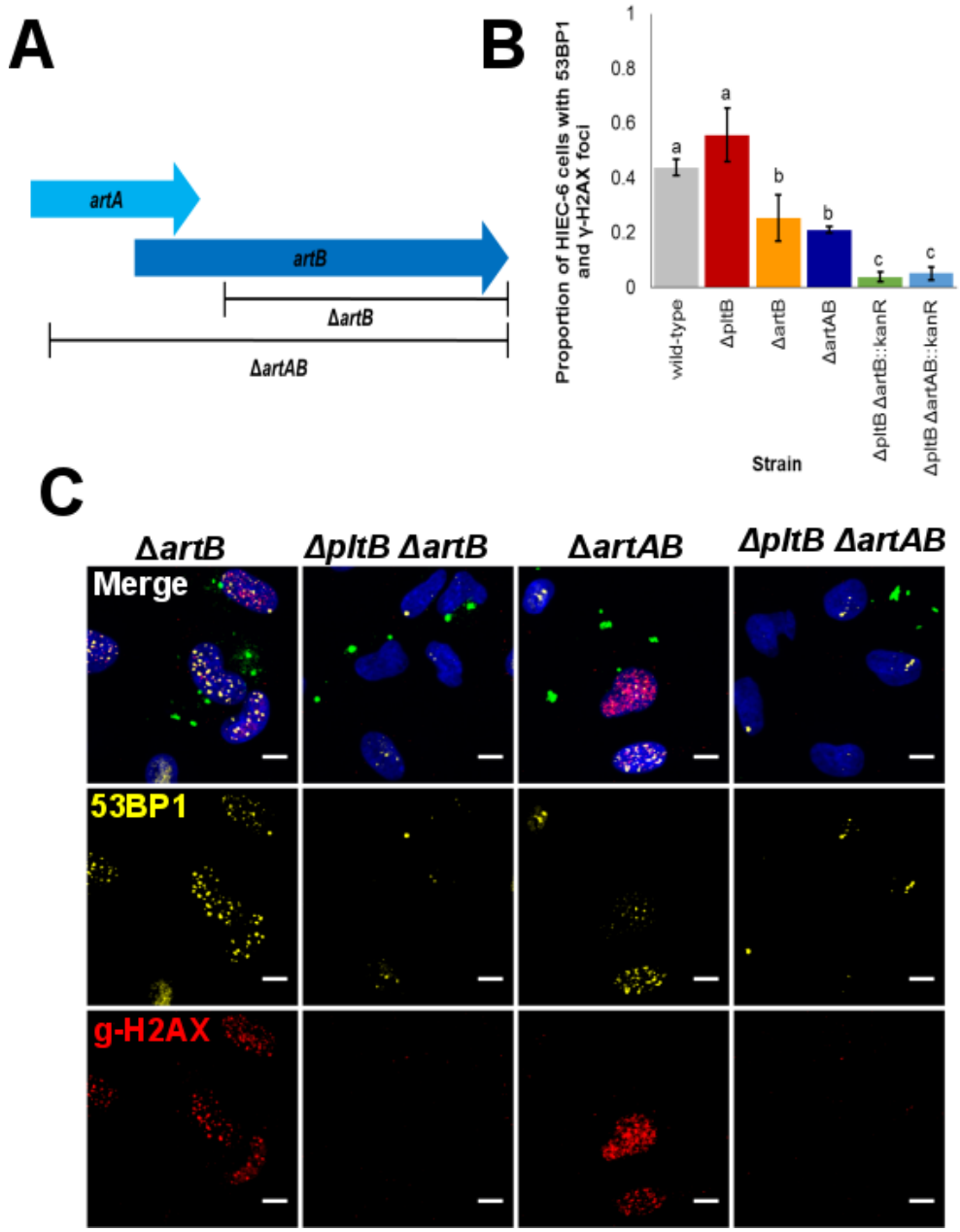

FIGURE 4.4 Strains require *artB* or *pltB* for S-CDT-mediated intoxication. Normal human intestinal epithelial cells (HIEC-6 cells) were infected with 2 x 10^6 *S.* Javiana strains harboring either single gene deletions in *artB, artAB,* or double deletions in *pltB* and either *artB* or *artAB.* At 48 hpi, cells were stained for DDR foci γH2AX and 53BP1. A) Schematic of the gene deletions for Δ*artB* and Δ*artAB* strains. B) Quantification of proportions of cells with >four 53BP1 foci, which co-localized with γH2AX foci. Treatments which do not share letters have significantly different ($p < 0.05$) proportions of cells with 53BP1 foci that also co-localized with γH2AX foci. C) Representative immunofluorescence images of HIEC-6 cells infected with *S.* Javiana strains. Green represents *Salmonella* cells, yellow foci represent 53BP1 foci, and γH2AX foci are shown in red.

To determine if ArtB could substitute for PltB as the binding subunit, we compared the amino acid sequences of ArtB with PltB, and mapped the 5 amino acid residues which are predicted to interact with host receptors for toxin binding to host cell surfaces (21). The Ser35 residue which is essential for binding to host cells (21) is present in ArtB from *S.* Javiana (Fig 4.5A). Similarly, the other amino acid residues are either perfectly aligned, or are offset by just 1 amino acid residue, suggesting that ArtB has the 5 key binding amino acid residues (Fig 4.5 A). Furthermore, the predicted 3D structures of the PltB and ArtB subunits from *S.* Javiana were structurally similar (Fig. 4.5 B), supporting the hypothesis that ArtB, could serve as a monomer for the pentameric binding subunit of the holotoxin.

FIGURE 4.5 Key amino acid residues necessary for binding to sugar moieties are conserved in ArtB. A) Clustal-w alignments of translated amino acid sequences of PltB and ArtB from *S.* Javiana strain CFSAN0001992. Letters highlighted in green represent key residues that are conserved, letters highlighted in red represent residues that differ between PltB and ArtB, but are next to letters in orange which show amino acid residues that interact with host glycans (21); "*" represents amino acid residues common to all three sequences (26).B) Predicted 3D structure of PltB and ArtB based off of translated amino acid sequences of *pltB* and *artB* extracted from *S.* Javiana strain CFSAN0001992, generated using SWISS-MODEL online software (27).

***artB* by is encoded by other S-CDT positive *Salmonella* serotypes.** ArtB (for ADP-ribosylation toxin subunit B) was first described for *S. enterica* subsp. *enterica* serotype Typhimurium (20), a S-CDT-negative serotype (8). To determine if *artB* was conserved among S-CDT-positive and negative nontyphoidal serotypes, we aligned the predicted amino acid sequence of ArtB from *S.* Typhimurium with that of predicted ArtB amino acid sequences from other S-CDT positive serotypes. Predicted amino acid sequences of ArtB from S-CDT-positive serotypes Minnesota, Javiana, Rubislaw, Montevideo, and Schwarzengrund were highly similar, with just 2 out of 141 amino acids different (98.6% identical). Similarly, predicted amino acid sequences for ArtB from Typhi CT-18 and Paratyphi ATCC 11511 strain had just 1 amino acid different (99.3% identical). The predicted ArtB amino acid sequence from *S.* Typhimurium DT 104 was the most variable (73.9% identical to ArtB from NTS serotypes in the data set, 73.2% identical to ArtB from typhoidal and paratyphoidal strains included, which agrees with what has been reported previously (6).

Because PltB was previously reported to be homologous to the B1 subunit of the subtilase cytotoxin produced (abbreviated Sub B1) by some strains of *E. coli* (4), we also aligned the predicted amino acid sequences of ArtB, PltB, and Sub B1. PltB and Sub B1 subunits were more conserved (48.2% identical), compared to ArtB aligned with Sub B1 (36.8% identical), and PltB aligned with ArtB (34.0% identical).

DISCUSSION

The ‘typhoid toxin’, characterized as an important virlunece factor for serotype Typhi, was recently found to be produced by nontyphoidal serotypes such as *S.* Javiana. The toxin’s role in nontyphoidal salmonellosis remains unclear. In this study, we determined which genes in the S-CDT islet are essential for S-CDT activity *in vitro,* for nontyphoidal serotype *S.* Javiana. We

also show that *S.* Javiana encodes multiple homologs of the binding subunit of S-CDT, and that deletion of both genes is necessary to abolish S-CDT-mediated intoxication.

To date, very few studies have been performed which characterize the role of genes in the S-CDT islet, and most have been performed using serotype Typhi. Multiple studies have confirmed that *cdtB*, encoding the active subunit of the toxin, is essential for the G2/M arrest and activation of the host's DDR, in both typhoidal and nontyphoidal serotypes (4, 6, 8, 9, 13, 22). Similarly, other studies have shown that *pltA*, encoding the ADP-ribosylating subunit which serves as a molecular anchor that mediates the interaction of CdtB with the binding subunit PltB (4), is also essential for S-CDT-mediated intoxication (3, 9). Deletion of *pltB*, encoding the binding subunit PltB, in *S.* Typhi is sufficient to abolish its ability to induce a G2/M cell cycle arrest (3); this does not appear to be true for *S.* Javiana though, as the *ΔpltB S.* Javiana strains used both in this study, and reported by Mezal *et al*.(9), retained their activity *in vitro*, suggesting that PltB is necessary for S-CDT intoxication by *S.* Typhi, but not *S.* Javiana. Deletion of accessory genes *STY1887* and *ttsA*, did not have a significant impact on the activity of S-CDT *in vitro* in this study. In contrast, in *S.* Typhi, *ttsA* was characterized as an N-acetyl-β-D-muramidase, and was shown to be essential for export of the toxin out of the host cell, but deletion of *STY1887* did not have any effect (19). Although a different cell type was used in that study (Henle cells vs. HIEC-6 cells in this study) it is unlikely that this alone may account for the observed differences, as both cell lines are of human origin. Together, these results suggest that the S-CDT produced by nontyphoidal serotypes such as *S.* Javiana, may utilize different mechanisms for S-CDT excretion, binding, and trafficking, compared to the S-CDT produced by *S.* Typhi.

Deletion of *artB* alone resulted in a significant decrease in cytotoxic activity (Fig 4.4) compared to wild-type. Because deletion of both *pltB* and *artB* was required to achieve proportions of HIEC-6 cells with activated DDR responses that were similar to uninfected control cells, it is likely that for *S.* Javiana, both ArtB and PltB can be incorporated into the S-CDT holotoxin. The conservation of key amino acid residues in ArtB, which have been shown to bind to host glycoproteins for PltB (4, 21), suggests that ArtB could potentially bind to host cells. Deletion of *pltA*, encoding PltA which serves as the physical anchor between CdtB and the PltB pentamer (4), abolished activity *in vitro*, this suggests that the binding pentamer is essential for S-CDT, further supporting the hypothesis that in the absence of *pltB*, *artB* could serve as a substitute. Alternatively, due to the significant decrease in cytotoxicity observed among HIEC-6 cells infected with Δ*artB* strains, it could be that *S.* Javiana uses both ArtB and PltB monomers for the monomer. Interestingly, *S.* Typhi strains CT-18 and Ty2 both encode *artB*, and alignments with *artB* from nontyphoidal serotypes show that these are highly conserved. It is unknown whether the Typhi strain used by Spano *et al.* (3) also encodes *artB*, which would account for why in that strain *pltB* is essential for S-CDT activity.

Alternatively, ArtB could facilitate binding to alternative molecular targets, either providing flexibility with binding to known cells, or expanding the variety of cell types that may be bound by the toxin. Deng *et al*. suggested that S-CDT binds to Neu5Ac-terminal glycans which are unique to humans due to a loss of function in the enzyme catalyzing the conversion of Neu5Ac to Neu5Gc, therefore supporting the hypothesis that the typhoid toxin is a host-adapted toxin produced by the host-restricted serotype (21). Future studies examining the potential for ArtB to substitute for PltB, or potentially, for the binding subunit to be comprised of both PltB

and ArtB monomers, will be essential to determining how ArtB contributes to S-CDT-mediated intoxication *in vitro*.

Overall, this study provides the first report of S-CDT islet and accessory genes, and their role in S-CDT-mediated intoxication in a normal epithelial cell model of infection. These results support that the S-CDT produced by nontyphoidal serotypes, may use multiple genes encoding homologs of the binding subunit. Furthermore, there are key differences in the requirement of genes *pltB* and *ttsA* for S-CDT-mediated intoxication for *S.* Typhi and the nontyphoidal serotype *S.* Javiana, suggesting that these serotypes differ mechanistically in the production, trafficking, or activity, of S-CDT *in vitro*.

MATERIALS AND METHODS

Bacterial strains, human cell lines, and culturing conditions. *Salmonella* strains (see Table 4.1) were stored at -80°C in 15% v/v glycerol. *Salmonella* were grown on brain heart infusion (BHI; Becton Dickinson, Sparks, MD) agar plates, which were incubated at 37°C. For infections, single colonies of *Salmonella* isolates grown on BHI agar plates were inoculated into 5-ml aliquots of LB (pH 8; 0.3 M NaCl), followed by incubation under static conditions at 37°C for 12 - 14 h. These cultures were subsequently sub-cultured 1:100 into fresh aliquots of LB (pH 8; 0.3 M NaCl), followed by incubation at 37°C under static conditions, until mid-log (OD_{600} = 0.4 – 0.5).

TABLE 4.1 Strains used in this study.

Strain ID	Genotype
FSL S5-0395	Wild-type
FSL M8-0532	S5-0395 Δ*pltA*
FSL M8-0533	S5-0395 Δ*pltB*
FSL M8-0540	S5-0395 Δ*cdtB*
FSL M8-0578	S5-0395 Δ*STY1887*
FSL M8-0577	S5-0395 Δ*ttsA*
FSL M8-0582	S5-0395 Δ*artB*
FSL M8-0583	S5-0395 Δ*artAB*
FSL M8-0588	S5-0395 Δ*phoN*::*kanR*
FSL M8-0587	S5-0395 Δ*cdtB* Δ*phoN*::*kanR*
FSL M8-0585	S5-0395 Δ*pltB* Δ*artB*::*kanR*

Human intestinal epithelial cells (HIEC-6 cells; ATCC) were grown in Opti-MEM media supplemented with recombinant epidermal growth factor (10 ng/mL; Gibco-Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco-Invitrogen), and were incubated at 37°C with 5% CO_2. Cells were tested every 3 months for *Mycoplasma* infection using the VenorGEM *Mycoplasma* detection kit (Sigma-Aldrich). For infections, HIEC-6 cells were grown in 6-well or 24-well plates (Corning, Corning, NY). Cells were seeded into 6-well (2×10^5 cells per well) or 24-well plates (1×10^5 cells) 48 $\pm$ 4 hours before infection.

Construction of Gene Deletions. Bacterial strains are shown in Table 2. All deletions were performed using the λ Red Recombinase system to create in-frame deletions (23). Briefly, FSL S5-0395 (*S.* Javiana wild-type) containing the pKD46 plasmid (described in (23)) was grown shaking at 30°C in LB broth containing 100 μg/mL ampicillin and 0.01% L-Arabinose (Sigma), to an optical density at 600 nm (OD_{600}) of 0.5 - 0.7. Cells were transformed with kanamycin

resistance cassettes containing flanking regions homologous to chromosomal sites homologous to the gene targeted for deletion, using electroporation followed by a 2 h recovery in SOC media (NEB) at 37°C. PCR amplification of kanamycin resistance cassettes were generated with high-fidelity polymerase Q5 (NEB; Ipswich, MA) was used to amplify kanamycin cassettes from pKD46, using primers listed in Table 4.2. Subsequently, cells were plated on LB supplemented with 50 μg/mL kanamycin and were incubated at 37°C for 18 – 24 h. Successful chromosomal integration of the kanamycin resistance cassette was confirmed by colony PCR. For removal of the kanamycin resistance cassette, cells were grown to mid-log phase (OD_{600} ~0.5- 0.7) and were electroporated with pCP20, followed by incubation at 30°C for 2 h; colonies were selected by plating on LB agar supplemented with 100 μg/mL ampicillin for 20 – 24 h. For strains requiring multiple deletions, crosses with phage P22 were conducted. In-frame deletions were confirmed by Sanger sequencing of PCR products amplifying upstream and downstream of the region deleted.

TABLE 4.2 Primers used in this study

Primer Name	Purpose	Primer sequence (5' to 3')
RM91cdtBKanup	Deletion of *cdtB*	AACACATATATCATTCAGATAAAAAAGTAATAATCGGGAGAGTAGATATCGTGTAGGCTGGAGCTGCTTC
RM92cdtBKandown	Deletion of cdtB	TATTCTGCACCTTACGCTCAAAGTACATGTCGTCAACGCTATTTACTCACATATGAATATCCTCCTTAG
RM97cdtBup_F	Confirm deletion of *cdtB*	CAACGTCATGAAACAATGGGTTATG
RM96cdtBdown_R	Confirm deletion of cdtB	ATATTCTGCACCTTACGCTCAAAGTAC
RM174PcdtBGFP_EcoRI_F	Complementation of *cdtB*	TAAGCAGAATTCTAAGTCACCTGTTTTGTGTTGAG
RM199_artBupstreamF	Confirm deletion of artB	CTTCCGCCGTCTGTTGCTTA
RM200_artBdownstreamR	Confirm deletion of artB	GGTGACAGTATCTGCGGCATTGTT
RM209_STY1887kanF	Deletion of *STY1887*	AATTATGAGTTGTTTTACCAGTCCAGCAATTATGGTGTAGGCTGGAGCTGCTTC
RM210_STY1887kanR	Deletion of *STY1887*	TTAATTATGTTGTATGGTATGAGAATGATAGTATTTCATATGAATATCCTCCTTAG
RM211_STY1887F	Confirm deletion of STY1887	GTACTTTGAGCGTAAGGTGCA
RM212_STY1887R	Confirm deletion of STY1887	TGGCGGTTTGTGATGATAGCATA
RM213_ttsAkanF	Deletion of *ttsA*	TTACAACCTTACCCGTTCCTTTATCCATCCGTGTAGGCTGGAGCTGCTTC
RM214_ttsAkanR	Deletion of *ttsA*	ATCTTTGCAGCTATCCTTAGTAGAGAAGGTGGTTACCATATGAATATCCTCCTTAG
RM215_ttsAF	Confirm deletion of *ttsA*	GTGGCTTTCACCAGTTCTCT
RM216_ttsAR	Confirm deletion of *ttsA*	GATGCAAGACCTGTAATAGAACTT
RM221_artABkanF	Deletion of *artAB*	ATGCAGTTACAAAGTGAGTATGTATCTGTAGTGTAGGCTGGAGCTGCTTC
RM222_cdtBcomp_AatII_R	Complementation of *cdtB*	GACGTCCATGTCGTCAACGCTATTTACTCA
RM223_artBintF	Screen for *artB*	GCTCTTGCGTCATTATCCAGTGTT
RM224_artBintR	Screen for artB	CACTTTACACATGGCATTGACACTAA
RM225_artB2kanF	Deletion of *artB*	AGTAATGTTCAGATTAAAAACCTGTCTTATGGTGTGTAGGCTGGAGCTGCTTC
RM226_artB2kanR	Deletion of *artB*	CCTGTTCTTTAGAGCGTTTAATACCCAGCAACATCATATGAATATCCTCCTTAG

***Salmonella* infection of HIEC-6 cells.** Treatments (i.e. strain used for infection, negative or positive control treatments) were randomly assigned to HIEC-6 cells seeded in 6-well or 24-well plates. HIEC-6 cells were infected with approximately 2 x 10^6 cfu or 4 x 10^6 cfu *Salmonella*, for HIEC-6 cells seeded in 24-well and 6-well plates, respectively. After incubation of the infected cells for 1 h at 37°C (with 5% CO_2), HIEC-6 cells were washed 3 times with phosphate buffered saline (PBS), followed by incubation with media supplemented with 100 μg/ml gentamicin (Gibco) for 1 h at 37°C to kill extracellular bacteria. Subsequently, HIEC-6 cells were washed an additional 3 times with PBS, and were then maintained in media containing 10 μg/ml gentamicin (Gibco) to prevent recurrent infection and bacterial outgrowth during incubation.

Flow Cytometry. Cell cycle analyses were performed on populations of HIEC-6 cells that were infected with *Salmonella* strains as described above, in order to determine the role of infection with S-CDT-positive strains on cell cycle progression. Briefly, HIEC-6 cells were washed once with PBS, and were harvested using 0.25% Trypsin EDTA (Gibco). Cells were fixed in ice-cold 70% ethanol, and were stored at -20°C. Ethanol-fixed cells were permeabilized with PBS containing 0.1% Tween-20 (PBS-T) and bovine serum albumin (1 g/100 ml) (Sigma-Aldrich, St. Louis, MO) at room temperature for 10 min. Cells were subsequently stained (10 min at room temperature) with a solution containing propidium iodide (PI; ThermoFisher Scientific, Waltham, MA) at a final concentration of 50 μg/ml and RNase A (Sigma-Aldrich) at a final concentration of 100 μg/ml. Stained cells were held at 4°C for no longer than 4 h, prior to DNA content analysis using the BD FACSARIA. Cells were gated to exclude doublets and multiplets, as described previously (24).

Immunofluorescence Staining. Immunofluorescence staining for γ-H2AX and 53BP1 foci was performed at 48 $\pm$ 2 h post infection (hpi) as described previously (8). Briefly, HIEC-6 cells

grown on 12 mm coverslips (ThermoFisher Scientific) were washed with PBS and were then fixed with 4% formaldehyde in PBS at room temperature for 10 - 15 min. Fixed cells were permeabilized with 0.1% Triton-X 100 in PBS at room temperature for 10 min. Permeabilized cells were blocked with 10% v/v normal donkey serum (Sigma) in PBS containing 0.1% Tween-20 (PBS-T; Sigma) for 1 h at room temperature. Incubation with primary and secondary antibodies was performed for 1 h at room temperature using the following dilution factors in PBS-T: polyclonal goat anti-*Salmonella* antibody (KPL antibodies, Gaithersburg, MA; 1:500), mouse anti-γ-H2AX (EMD Millipore, Billerica, MA; 1:500), rabbit anti-53BP1 (Novus Biologicals, Littleton, CO; 1:500). Incubation with secondary antibodies diluted 1:200, except where noted, in PBS-T was performed for 1 h at room temperature; donkey anti-goat conjugated to Alexa 488; donkey anti-rabbit conjugated to Alexa 555 (diluted 1:500); and donkey anti-mouse conjugated to Alexa 647 (all ThermoFisher Scientific). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) at a final concentration of 1 μg/ml for 5 min at room temperature. Slides were mounted onto microscope slides with glycergel (Dako, Carpinteria, CA) and were imaged using a Zeiss 710 confocal microscope. Images were processed with FIJI software, and cells were counted using the cell counter plug-in (25). At least 50 nuclei were analyzed per slide to identify cells that had more than four 53BP1 positive foci and were also positive for γH2AX; these cells were designated as 53BP1 and γH2AX positive cells (or "double positive cells"). The observer was blinded to the treatment while collecting and analyzing images.

Statistical Analysis. All statistical analyses were performed using R software (version 3.0.2; R-project, Vienna, Austria). Linear mixed effects models were developed to determine significant associations between strains (i.e. genotypes) and either (i) proportions of cell populations in

G2/M, or (ii) proportions of cells with an activated DDR as determined using immunofluorescence staining for 53BP1 and γH2AX foci. Strain was included as a fixed effect, while date of experiment was included in models as a random effect. Data were transformed by taking the square root or performing a rank transformation when appropriate to obtain a normal distribution of the residuals. Figs were constructed using the untransformed data. The lsmeans (least square means) package was used to perform pairwise comparisons among treatments, and P-values were adjusted using the Tukey method to correct for multiple comparisons.

REFERENCES

1. Majowicz SE, Musto J, Scallan E, Angulo FJ, Kirk M, O'Brien SJ, Jones TF, Fazil A, Hoekstra RM. 2010. The global burden of nontyphoidal *Salmonella* gastroenteritis. Clinical Infectious Diseases 50:882-889.
2. Kirk MD, Pires SM, Black RE, Caipo M, Crump JA, Devleesschauwer B, Döpfer D, Fazil A, Fischer-Walker CL, Hald T, Hall AJ, Keddy KH, Lake RJ, Lanata CF, Torgerson PR, Havelaar AH, Angulo FJ. 2015. World Health Organization Estimates of the Global and Regional Disease Burden of 22 Foodborne Bacterial, Protozoal, and Viral Diseases, 2010: A Data Synthesis. PLoS Med 12:e1001921.
3. Spanò S, Ugalde JE, Galán JE. 2008. Delivery of a *Salmonella* Typhi exotoxin from a host intracellular compartment. Cell host & microbe 3:30-38.
4. Song J, Gao X, Galán JE. 2013. Structure and function of the *Salmonella* Typhi chimaeric A2B5 typhoid toxin. Nature 499:350-354.
5. den Bakker HC, Switt AIM, Govoni G, Cummings CA, Ranieri ML, Degoricija L, Hoelzer K, Rodriguez-Rivera LD, Brown S, Bolchacova E. 2011. Genome sequencing reveals diversification of virulence factor content and possible host adaptation in distinct subpopulations of *Salmonella enterica*. BMC genomics 12:425.
6. Rodriguez-Rivera LD, Bowen BM, den Bakker HC, Duhamel GE, Wiedmann M. 2015. Characterization of the cytolethal distending toxin (typhoid toxin) in non-typhoidal *Salmonella* serovars. Gut pathogens 7:1.
7. Miller R, Wiedmann M. 2016. Dynamic Duo—The Salmonella Cytolethal Distending Toxin Combines ADP-Ribosyltransferase and Nuclease Activities in a Novel Form of the Cytolethal Distending Toxin. Toxins 8:121.
8. Miller RA, Wiedmann M. 2016. The Cytolethal Distending Toxin Produced by Nontyphoidal *Salmonella* Serotypes Javiana, Montevideo, Oranienburg, and Mississippi Induces DNA Damage in a Manner Similar to That of Serotype Typhi. mBio 7:e02109-02116.
9. Mezal EH, Bae D, Khan AA. 2014. Detection and functionality of the CdtB, PltA, and PltB from *Salmonella enterica* serovar Javiana. Pathogens and disease 72:95-103.
10. Dixon SD, Huynh MM, Tamilselvam B, Spiegelman LM, Son SB, Eshraghi A, Blanke SR, Bradley KA. 2015. Distinct Roles for CdtA and CdtC during Intoxication by Cytolethal Distending Toxins. PLoS ONE 10:e0143977.
11. Gargi A, Tamilselvam B, Powers B, Prouty MG, Lincecum T, Eshraghi A, Maldonado-Arocho FJ, Wilson BA, Bradley KA, Blanke SR. 2013. Cellular interactions of the cytolethal distending toxins from *Escherichia coli* and *Haemophilus ducreyi*. Journal of Biological Chemistry 288:7492-7505.
12. Nešić D, Hsu Y, Stebbins CE. 2004. Assembly and function of a bacterial genotoxin. Nature 429:429-433.
13. Haghjoo E, Galán JE. 2004. *Salmonella* Typhi encodes a functional cytolethal distending toxin that is delivered into host cells by a bacterial-internalization pathway. Proceedings of the National Academy of Sciences of the United States of America 101:4614-4619.
14. Belluz LDB, Guidi R, Pateras IS, Levi L, Mihaljevic B, Rouf SF, Wrande M, Candela M, Turroni S, Nastasi C. 2016. The Typhoid Toxin Promotes Host Survival and the Establishment of a Persistent Asymptomatic Infection. PLoS Pathog 12:e1005528.

15. Boore AL, Hoekstra RM, Iwamoto M, Fields PI, Bishop RD, Swerdlow DL. 2015. *Salmonella enterica* infections in the United States and assessment of coefficients of variation: a novel approach to identify epidemiologic characteristics of individual serotypes, 1996–2011. PloS one 10:e0145416.
16. Sabbagh SC, Forest CG, Lepage C, Leclerc J-M, Daigle F. 2010. So similar, yet so different: uncovering distinctive features in the genomes of *Salmonella enterica* serovars Typhimurium and Typhi. FEMS microbiology letters 305:1-13.
17. Atif SM, Winter SE, Winter MG, McSorley SJ, Bäumler AJ. 2014. *Salmonella enterica* serovar Typhi impairs CD4 T cell responses by reducing antigen availability. Infection and immunity 82:2247-2254.
18. Winter SE, Winter MG, Atluri V, Poon V, Romão EL, Tsolis RM, Bäumler AJ. 2015. The flagellar regulator TviA reduces pyroptosis by *Salmonella enterica* serovar Typhi. Infection and immunity 83:1546-1555.
19. Hodak H, Galan JE. 2013. A *Salmonella* Typhi homologue of bacteriophage muramidases controls typhoid toxin secretion. EMBO Rep 14:95-102.
20. Saitoh M, Tanaka K, Nishimori K, Makino S-i, Kanno T, Ishihara R, Hatama S, Kitano R, Kishima M, Sameshima T. 2005. The *artAB* genes encode a putative ADP-ribosyltransferase toxin homologue associated with *Salmonella enterica* serovar Typhimurium DT104. Microbiology 151:3089-3096.
21. Deng L, Song J, Gao X, Wang J, Yu H, Chen X, Varki N, Naito-Matsui Y, Galán JE, Varki A. 2014. Host adaptation of a bacterial toxin from the human pathogen *Salmonella* Typhi. Cell 159:1290-1299.
22. Williams K, Gokulan K, Shelman D, Akiyama T, Khan A, Khare S. 2015. Cytotoxic mechanism of cytolethal distending toxin in nontyphoidal *Salmonella* serovar (*Salmonella* Javiana) during macrophage infection. DNA and cell biology 34:113-124.
23. Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A 97:6640-6645.
24. Wersto RP, Chrest FJ, Leary JF, Morris C, Stetler-Stevenson M, Gabrielson E. 2001. Doublet discrimination in DNA cell-cycle analysis. Cytometry 46:296-306.
25. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B. 2012. Fiji: an open-source platform for biological-image analysis. Nature methods 9:676-682.
26. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 25:4876-4882.
27. Arnold K, Bordoli L, Kopp J, Schwede T. 2006. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics 22:195-201.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

CONCLUSIONS

Not all *Salmonella* serotypes cause the same disease. The discovery of the typhoid toxin in serotype Typhi represented an important advancement in the understanding of the biology of typhoid fever, demonstrating that the toxin plays a role in the resulting disease. Furthermore, characterization of this same toxin in nontyphoidal serotypes has shown that S-CDT plays an important role in pathogenesis at the cellular level.

Nontyphoidal serotypes produce a S-CDT that acts in a similar manner to the typhoid toxin produced by serotype Typhi. Importantly, the research presented here highlights that, among the 21 nontyphoidal serotypes causing the majority of clinical cases of salmonellosis in the US, 4 of these serotypes carry genes encoding S-CDT. Altogether, this accounts for an estimated 150,280 cases (90% C.I.: 111,812 – 196,954) of salmonellosis in the US each year which are caused by these S-CDT-positive serotypes. *In vitro*, infection of eukaryotic cells with S-CDT-positive serotypes results in activation of the DNA damage response, and a cell cycle arrest, both supporting the conclusion that infection with these serotypes results in DNA damage. Because these effects were also observed for uninfected cells in close proximity to infected cells, S-CDT is capable of acting via autocrine and paracrine pathways, therefore suggesting that exposure to S-CDT in the course of an infection with S-CDT-positive serotypes is widespread, as the cells do not have to be infected in order to exhibit signs of S-CDT intoxication.

Introduction of a new cellular model of S-CDT-mediated intoxication. One of the key limitations of previous studies characterizing the effect of S-CDT *in vitro*, was the use of cancer cell lines. While cancerous cell lines were beneficial for first establishing the activity of S-CDT, these cell lines often have defects in either DNA damage checkpoints or in DNA damage repair pathways, which therefore limits their use in determining the cellular outcome of the DNA

damage. To this end, one of the primary goals of this research was to establish that the S-CDT-mediated intoxication also occurs in non-cancerous cell lines. This represents an important next step in determining the fate of cells that sustain DNA damage following exposure to S-CDT.

Expanding the repertoire of S-CDT-associated genes and their importance *in vitro*. As the holotoxin is only comprised of the protein products of *cdtB*, *pltB*, and *pltA*, very few studies had been undertaken to investigate the role of *ttsA* and *STY1887* in S-CDT-mediated virulence (1). This research provides evidence that the S-CDT encoded by nontyphoidal serotype *S.* Javiana may have alternative requirements for trafficking and export, as deletion of *ttsA* did not abolish S-CDT activity, as was reported for serotype Typhi (1).

Despite the discovery of the PltB and PltA homologs ArtB and ArtA in serotype Typhimurium, their role in S-CDT-intoxication of nontyphoidal serotypes was unknown. The research described here provides the first evidence that ArtB influences S-CDT-induced intoxication *in vitro*. Future work involving proteomic methods to characterize the physical interactions between ArtB and S-CDT, as well as any potential differences in cell receptors bound by PltB versus ArtB will be beneficial in determining the true impact or requirement of nontyphoidal serotypes encoding multiple binding subunits for S-CDT.

FUTURE DIRECTIONS

This research provides an important framework for future investigations regarding the regulation of S-CDT, as well as its contributions to acute disease severity and any associated long-term sequelae. Given the large number of infections with S-CDT-positive serotypes, it will be important to better understand how S-CDT impacts the severity and outcome of infection. Furthermore, the transcriptional and translational regulation of S-CDT is poorly understood. Future investigations aimed at expanding our understanding of i) the transcriptional and

translational regulation of S-CDT, (ii) the role of S-CDT in the acute onset of disease, and contributions to disease severity, and (iii) the long-term effects of S-CDT exposure, will be beneficial for understanding the true influence of S-CDT in salmonellosis (Fig 5.1).

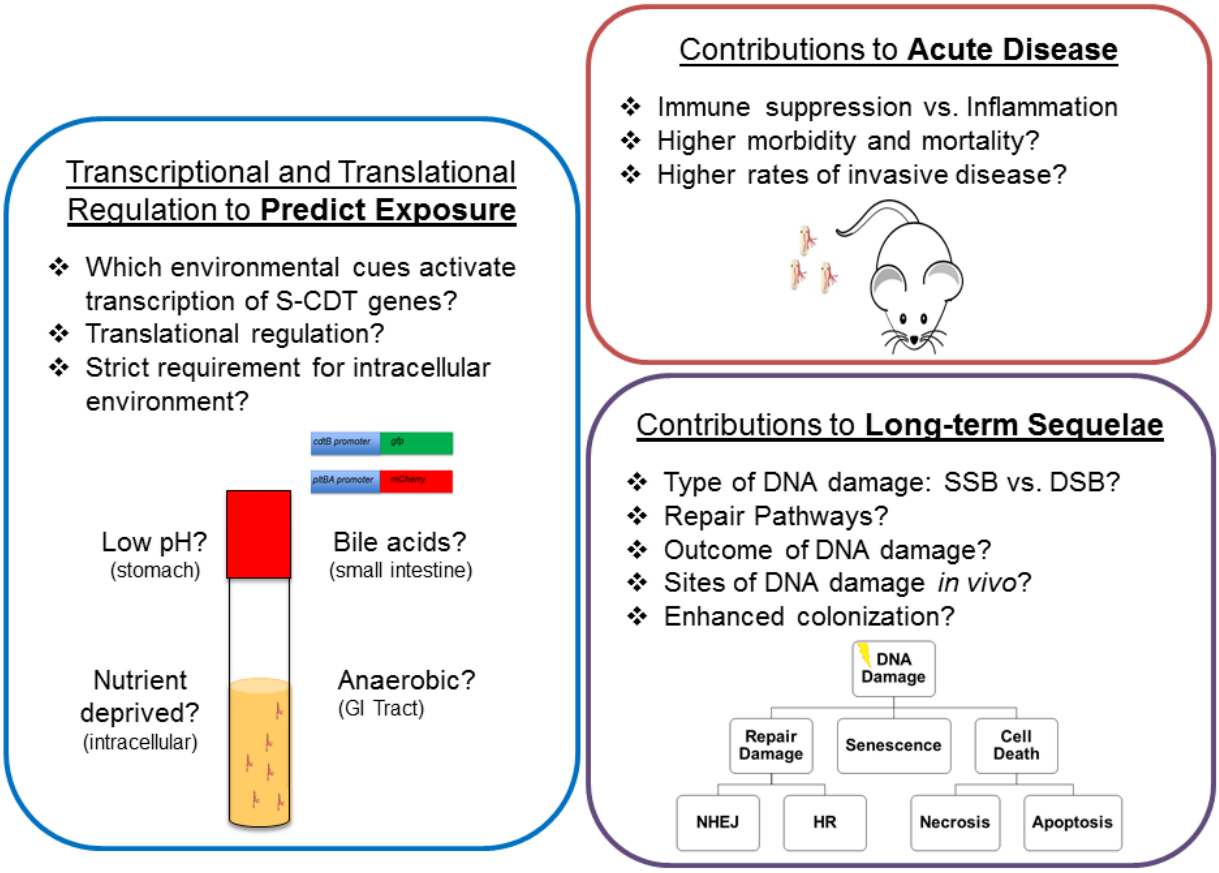

FIGURE 5.1. Possible future directions for S-CDT characterizations in nontyphoidal *Salmonella* serotypes. Abbreviations: Salmonella Cytolethal Distending Toxin (S-CDT); gastrointestinal tract (GI); single strand break (SSB); double strand break (DSB); non-homologous end joining (NHEJ); homologous recombination (HR).

Transcriptional and translational regulation of S-CDT for predicting anatomical regions most likely to be exposed to S-CDT. Transcriptional regulation of S-CDT has only been briefly characterized for serotype Typhi (1, 2). Due to the marked genomic differences between *S.* Typhi and other serotypes (3, 4), it is necessary to identify regulatory factors governing expression and translation of S-CDT for NTS serotypes. For serotype Typhi, transcription of *pltA*, *pltB*, and *cdtB*

(encoding the S-CDT toxin subunits PltA, PltB, and CdtB, respectively), only occurs when *S.* Typhi cells are located within the salmonella containing vacuole (SCV), approximately 5 hours post infection (5). This mirrors expression of salmonella pathogenicity island 2 (SPI-2) encoded genes (6), which are expressed when *Salmonella* cells are located within the SCV. It is likely that the same intracellular requirement is also true for S-CDT production for nontyphoidal serotypes as well, as co-incubation with supernatants of stationary phase cultures of *S.* Javiana fail to invoke a G2/M arrest and to activate the DNA damage response (DDR) in HeLa cells. Because relatively few *Salmonella* cells successfully invade host cells (7), this would limit the production and dissemination of the toxin. However, export of S-CDT outside of infected cells, would increase dissemination of the toxin, thereby increasing the potential exposure. Immunofluorescence staining clearly shows that DNA damage is not restricted to infected cells, and that intoxication with S-CDT may occur by paracrine pathways, as uninfected HeLa cells among populations of cells infected with *S.* Javiana also show an activated DDR. It is still unclear which environmental cues serve as the molecular signals governing transcription and translation of S-CDT subunits and associated proteins (i.e. TtsA and STY1887). Understanding the regulatory elements controlling expression of S-CDT would inform the anatomical regions which would be exposed to S-CDT during the course of an infection, and would guide *in vivo* analyses for examining S-CDT-mediated DNA damage.

S-CDT's contributions to acute salmonellosis. Analysis of rates of invasive disease among NTS serotypes suggests that infections with S-CDT-encoding serotypes are significantly more likely to result in invasive disease (8). For *S.* Typhi, S-CDT has been established as an important virulence factor, not only contributing to acute symptoms of typhoid fever (9), but also to immune suppression which enables prolonged infection and invasive disease in a mouse model

of infection (10). Due to the established link between S-CDT and the development of invasive disease and prolonged duration of infections with *S.* Typhi, it is likely that S-CDT plays an important role for NTS infections as well.

CDT production by other Gram-negative pathogens has been shown to be necessary for establishment of chronic infections with *Helicobacter hepaticus* (11) and the development of gastroenteritis for *Campylobacter jejuni* (12). Therefore, *in vivo* studies represent a necessary next step in characterizing the activity of, and implications of, S-CDT exposure accompanying infections with NTS serotypes.

Contributions to long-term sequelae- the outcome of DNA damage. S-CDT acts as a nuclease *in vitro*, causing a G2/M cell cycle arrest and activation of the DDR (13-15). The ultimate outcome of the DNA damage sustained (i.e. successful repair of damaged DNA, apoptosis, senescence, etc.), remains unknown. Analyses with *E. coli* CDT demonstrated that single strand breaks (SSB) are converted to double strand breaks (DSB) during the G2 cell cycle phase (16). The extent of DNA damage, including whether S-CDT induces SSB or DSB, or both, as well as the mechanisms by which this DNA are repaired, are currently unknown. DNA damage repair mediated by non-homologous end joining (NHEJ) is error prone, and occurs by excising nucleotides in order to join blunt ends (17). However, during S/G2 phases, when a suitable template is available, DNA damage repair can occur using homology directed repair, which is essentially error free (17). Characterizing how the DNA damage induced by S-CDT is repaired will be important for assessing the potential for carcinogenic transformation following S-CDT induced cytotoxicity. For *Salmonella*, it is still not clear if the DNA damage preferentially occurs in G2/M phases, or if the damage occurs throughout the cell cycle, but the damage is not being repaired. Since the cell cycle influences the repair pathway (18, 19), it is necessary to assert

when the damage is occurring, as this will provide important information about the potential for carcinogenic transformation.

Cancer-derived cell lines which have been used to characterize activation of the DDR following exposure to S-CDT, often have faulty DNA repair mechanisms, and therefore do not recapitulate normal DNA repair processes (reviewed in (20)). Therefore, use of untransformed cell lines to study the overall outcome of S-CDT induced DNA damage, represents an important step in assessing the implications of S-CDT DNA damage.

FOOD SAFETY REGULATORY IMPLICATIONS OF S-CDT PRODUCTION BY NONTYPHOIDAL SEROTYPES

Toxin-based classification of foodborne pathogens facilitates clinical treatment decisions and epidemiological investigations. Select foodborne pathogens are differentiated based on the presence of certain virulence factors for both epidemiological and clinical reasons. For example, shiga toxin producing *E. coli* (STEC) are characterized based on the presence of stx_1 and stx_2 genes encoding shiga toxins 1 and 2, respectively (21), as antibiotic treatment of STEC infections is associated with a significantly higher incidence of hemolytic uremic syndrome and is therefore discouraged (22). S-CDT status could influence treatment regimens, and could also serve as an epidemiological tool for comparing similar strains implicated with a common food vehicle, as is done with the *stx* genes in *E. coli* (21).

Foodborne pathogens as adulterants. According to 64 CFR 2803, foods containing select STEC serotypes are considered adulterated (23). While nontyphoidal *Salmonella* serotypes are not currently classified as adulterants, the potential genotoxic effect of S-CDT in the context of foodborne salmonellosis may merit future investigation and discussion regarding the status of S-CDT-producing nontyphoidal serotypes as adulterants.

Overall, S-CDT is best likened as a proverbial ‘Pandora’s Box’ – efforts to characterize and understand its’ regulation, structure-function, activity, and impact on hosts at both a cellular and organismal level, are still in their infancy, with each new discovery leading to a very exciting contribution to the S-CDT story.

REFERENCES

1. Hodak H, Galan JE. 2013. A *Salmonella* Typhi homologue of bacteriophage muramidases controls typhoid toxin secretion. EMBO Rep 14:95-102.
2. Haghjoo E, Galán JE. 2007. Identification of a transcriptional regulator that controls intracellular gene expression in *Salmonella* Typhi. Molecular microbiology 64:1549-1561.
3. Sabbagh SC, Forest CG, Lepage C, Leclerc J-M, Daigle F. 2010. So similar, yet so different: uncovering distinctive features in the genomes of *Salmonella enterica* serovars Typhimurium and Typhi. FEMS microbiology letters 305:1-13.
4. den Bakker HC, Switt AIM, Govoni G, Cummings CA, Ranieri ML, Degoricija L, Hoelzer K, Rodriguez-Rivera LD, Brown S, Bolchacova E. 2011. Genome sequencing reveals diversification of virulence factor content and possible host adaptation in distinct subpopulations of *Salmonella enterica*. BMC genomics 12:425.
5. Spanò S, Ugalde JE, Galán JE. 2008. Delivery of a *Salmonella* Typhi exotoxin from a host intracellular compartment. Cell host & microbe 3:30-38.
6. Zwir I, Latifi T, Perez JC, Huang H, Groisman EA. 2012. The promoter architectural landscape of the *Salmonella* PhoP regulon. Molecular microbiology 84:463-485.
7. Ackermann M, Stecher B, Freed NE, Songhet P, Hardt W-D, Doebeli M. 2008. Self-destructive cooperation mediated by phenotypic noise. Nature 454:987-990.
8. Rodriguez-Rivera LD, Bowen BM, den Bakker HC, Duhamel GE, Wiedmann M. 2015. Characterization of the cytolethal distending toxin (typhoid toxin) in non-typhoidal *Salmonella* serovars. Gut pathogens 7:1.
9. Song J, Gao X, Galán JE. 2013. Structure and function of the *Salmonella* Typhi chimaeric A2B5 typhoid toxin. Nature 499:350-354.
10. Belluz LDB, Guidi R, Pateras IS, Levi L, Mihaljevic B, Rouf SF, Wrande M, Candela M, Turroni S, Nastasi C. 2016. The Typhoid Toxin Promotes Host Survival and the Establishment of a Persistent Asymptomatic Infection. PLoS Pathog 12:e1005528.
11. Ge Z, Feng Y, Whary MT, Nambiar PR, Xu S, Ng V, Taylor NS, Fox JG. 2005. Cytolethal distending toxin is essential for *Helicobacter hepaticus* colonization in outbred Swiss Webster mice. Infection and immunity 73:3559-3567.
12. Fox JG, Rogers AB, Whary MT, Ge Z, Taylor NS, Xu S, Horwitz BH, Erdman SE. 2004. Gastroenteritis in NF-κB-deficient mice is produced with wild-type *Camplyobacter jejuni* but not with *C. jejuni* lacking cytolethal distending toxin despite persistent colonization with both strains. Infection and immunity 72:1116-1125.
13. Miller R, Wiedmann M. 2016. Dynamic Duo—The Salmonella Cytolethal Distending Toxin Combines ADP-Ribosyltransferase and Nuclease Activities in a Novel Form of the Cytolethal Distending Toxin. Toxins 8:121.
14. Jinadasa RN, Bloom SE, Weiss RS, Duhamel GE. 2011. Cytolethal distending toxin: a conserved bacterial genotoxin that blocks cell cycle progression, leading to apoptosis of a broad range of mammalian cell lineages. Microbiology 157:1851-1875.
15. Galán JE. 2016. Typhoid toxin provides a window into typhoid fever and the biology of *Salmonella* Typhi. Proceedings of the National Academy of Sciences:201606335.

16. Fedor Y, Vignard J, Nicolau-Travers ML, Boutet-Robinet E, Watrin C, Salles B, Mirey G. 2013. From single-strand breaks to double-strand breaks during S-phase: a new mode of action of the *Escherichia coli* Cytolethal Distending Toxin. Cell Microbiol 15:1-15.
17. Lieber MR. 2010. The mechanism of double-strand DNA break repair by the nonhomologous DNA end joining pathway. Annual review of biochemistry 79:181.
18. Chapman JR, Taylor Martin RG, Boulton Simon J. 2012. Playing the End Game: DNA Double-Strand Break Repair Pathway Choice. Molecular Cell 47:497-510.
19. Shibata A, Conrad S, Birraux J, Geuting V, Barton O, Ismail A, Kakarougkas A, Meek K, Taucher-Scholz G, Löbrich M. 2011. Factors determining DNA double-strand break repair pathway choice in G2 phase. The EMBO journal 30:1079-1092.
20. Lahtz C, Pfeifer GP. 2011. Epigenetic changes of DNA repair genes in cancer. Journal of molecular cell biology 3:51-58.
21. Gould LH, Bopp C, Strockbine N, Atkinson R, Baselski V, Body B, Carey R, Crandall C, Hurd S, Kaplan R. 2009. Recommendations for diagnosis of shiga toxin--producing *Escherichia coli* infections by clinical laboratories. MMWR Recommendations and reports: Morbidity and mortality weekly report Recommendations and reports/Centers for Disease Control 58:1-14.
22. Wong CS, Jelacic S, Habeeb RL, Watkins SL, Tarr PI. 2000. The risk of the hemolytic-uremic syndrome after antibiotic treatment of *Escherichia coli* O157:H7 infections. The New England journal of medicine 342:1930-1936.
23. Wheeler T, Kalchayanand N, Bosilevac JM. 2014. Pre-and post-harvest interventions to reduce pathogen contamination in the US beef industry. Meat science 98:372-382.